CHEMORESISTANCE MECHANISMS IN ACUTE MYELOID LEUKEMIA STEM CELLS

A Dissertation

Presented to the Faculty of the Weill Cornell Graduate School

Of Medical Sciences

In Partial Fulfillment of the Requirements of the Degree of

Doctor of Philosophy

by

Alec W. Stranahan

June 2017

CHEMORESISTANCE MECHANISMS IN ACUTE MYELOID LEUKEMIA STEM CELLS

Alec W. Stranahan, Ph.D.

Cornell University 2017

Acute myeloid leukemia (AML) is characterized by poor clinical outcome due to high rates of relapse driven by therapy-resistant leukemic stem cells (LSC). Despite major advances in our understanding of the mechanisms that drive AML pathogenesis, our comprehension of responses to therapy remains poor, and treatment strategies have not dramatically changed over the past thirty years. In this dissertation, we directly evaluated LSC responses to induction therapy by performing gene expression profiling of LSC and non-LSC blast populations from paired diagnostic:post-induction therapy patient samples. Our work identified 5-lipoxygenase (5-LO), amongst several other genes, as potential chemoresistance mediators in AML LSCs. Confirmation of the broad role of 5-LO in leukemia was observed through loss-of-function studies in 5-LO deficient models of myeloid leukemogenesis, which exhibit improved survival and responses to chemotherapy both *in vitro* and *in vivo*. In contrast, 5-LO deficient hematopoietic stem/progenitor cells exhibit enhanced recovery in response to myeloablative therapy. Genetic and pharmacologic perturbation of 5-LO activity confirms that the leukotriene-synthetic capacity of 5-LO is a requirement for its chemoprotective effects. Together, our data identify multiple novel regulators of LSC chemoresistance and highlight 5-LO as a target for therapies that seek to enhance the effect of induction therapy.

BIOGRAPHICAL SKETCH

Alec Stranahan received his Bachelor of Science degree with Honors from the University of Vermont in 2010. While completing his undergraduate studies, he utilized gene editing and biochemical approaches to investigate the translational regulation of Notch, a protein with described roles in the progression of Alzheimer's disease. Prior to starting his Ph.D., Alec worked as a research assistant at Mount Sinai Medical Center, where he strived to optimize the *ex vivo* differentiation of human embryonic stem cells into functional red blood cells. This work has therapeutic implications for the treatment of sickle cell anemia and beta-thalassemia, as well as expanding knowledge of globin switching during red cell development.

During his graduate training at Weill Cornell Medicine, Alec deepened his understanding of stem cells within the hematopoietic system to include both normal and malignant hematopoietic stem cells, developing targeted therapies for the treatment of acute myeloid leukemia. His work has resulted in numerous presentations at both national and international science conferences, with two manuscripts under preparation. Alec is the recipient of the T32 training award in Molecular Biology, the TL1 translational research grant, and the 2017 recipient of the Lee Family Scholarship from Weill Cornell, which will provide full tuition support for accelerated MBA training upon graduation. Alec seeks to combine the scientific expertise gained during his Ph.D. with business concepts to drive future clinical drug development.

This work is dedicated to my family and friends who
provided support, encouragement, and levity
during this influential time in my life —
it would not have been possible without you

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my primary mentor, Dr. Christopher Park, for his guidance and patience ever since I joined the lab several years ago. I have appreciated his unwavering support in whatever I have set my mind to, both scientifically and professionally. Chris consistently pushed me to be better than I am and I believe I have emerged a stronger person because of it.

I would also like to thank the members of my thesis committee, Drs. Neal Rosen, Ross Levine, and Kirk Deitsch for their illuminating input at key points throughout my thesis work. Our conversations over the years have contributed additional depth to my research. I would like to acknowledge Drs. Ari Melnick and Ross Levine, who are co-mentors on my TL1 translational science grant, Dr. Anthony Brown who was my mentor on the MCB T32 training grant, and Dr. Dean Bajorin with whom I have met regularly as my Associate Program Director at the Weill Cornell Clinical and Translational Science Center.

I am grateful to the many collaborators who contributed to this work, especially Dr. Faye Feller who initiated the chemoresistance target validation experiments and helped me hit the ground running during my rotation and early days in the Park Lab. I would also like to thank Drs. Wenhuo Hu and Sean Devlin at Memorial Sloan-Kettering Cancer Center for their assistance in analyzing the patient transcriptome data.

I would additionally like to acknowledge the MSKCC Shared Services, particularly the Flow Cytometry Core, Integrated Genomics Operation, and Molecular Cytology Core which were indispensable for completion of this research. My work was also supported by the National Institutes of Health grant 5T32GM008539 for pre-

doctoral training in Molecular Biology and the National Center for Advancing Translational Sciences (NCATS) grant TL1TR000459 of the Clinical and Translational Science Center at Weill Cornell Medical College.

On a more personal note, I would like to thank all members of the Park Lab, both past and present, for their kindness and relentless generosity in sharing both their knowledge and time. I would particularly like to acknowledge Sarah Qamar and Mona Khalaj from the Park Lab, as well as the many other close friends I've made during my Ph.D., who made me smile every day and kept my sanity intact. I have accumulated many great memories during my Ph.D., which is a reflection of the wonderful people I've met. Finally, I would like to thank my family for their endless support and for being proud of me no matter what I set out to accomplish. I hope to continue to make you proud far into the future.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

5-FU	5-flourouracil
5-LO	5-lipoxygenase
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APL	Acute promylocytic leukemia
Ara-C	Cytarabine
ATCC	American Type Culture Collection
ATRA	All-*trans* retinoic acid
BM	Bone marrow
CB	Umbilical cord blood
CBC	Complete blood count
CCl5	CC-chemokine ligand
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitors
COX	Cyclooxygenase
CR	Complete remission
CRi	Complete remission with incomplete recovery
CRm	Molecular complete remission
CSF	Colony-stimulating factor
CST7	Cystatin F
cysLT	Cysteinyl leukotriene
DLL4	Delta-like ligand 4
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DNR	Daunorubicin
Doxo	Doxorubicin
Dox	Doxycycline
DSMZ	Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH
E21	Embryonic day 21
ECOG	Eastern Coopoerative Oncology Group
ELDA	Extreme Limiting Dilution Analysis
EV	Empty vector
FAB	French-American-British
FACS	Flourescence-activated cell sorting
FC	Fold-change
FDA	Food and Drug Administration
FLAP	5-LO activating protein

GEXC	Gene Expression Commons
GMP	Granulocyte-macrophage progenitors
GPCR	G-protein coupled receptor
GSEA	Gene set enrichment analysis
Hh	Hedgehog
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HSPC	Hematopoietic stem and progenitor cell
IFN	Interferon-α
KO	5-LO Knockout
L-GMP	Leukemic granulocyte-macrophage progenitor
LIC	Leukemia-initiating cell
LSC	Leukemic stem cells
LSK	$Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$
LYZ	Lysozyme
MDS	Myelodysplastic syndrome
MEP	Megakaryocyte-erythroid progenitors
MLL	Mixed lineage-leukemia
MNC	Mononuclear cell
MRD	Minimal residual disease
Msi2	Musashi-2
NF-kB	Nuclear factor kB
NK	Natural killer
OS	Overall survival
P-gp	P-glycoprotein
PCA	Principle component analysis
PCR	Polymerase chain reaction
RBC	Red blood cell
RNA	Ribonucleic acid
RQ	Relative transcript quantity
MDR	Multi-drug resistance
SCF	Stem cell factor
SEM	Standard error mean
shRNA	Short hairpin RNA
TCGA	The Cancer Genome Atlas
TPO	Thrombopoietin
TSP-1	Thrombospondin-1
WHO	World Health Organization
WT	Wild-type

CHAPTER ONE:
INTRODUCTION

Beginning in early gestation, all cell types of the blood are specified in a hierarchical manner originating from a population of pluripotent hematopoietic stem cells (HSCs) through a process known as hematopoiesis. Perturbations in this process result in a variety of hematological malignancies, including acute myeloid leukemia (AML), which arises due to a block in myeloid differentiation, resulting in an accumulation of leukemic blast cells. As leukemic blasts are rapidly dividing, the majority of the tumor burden can be debulked using standard induction chemotherapy. However, chemotherapy is relatively ineffective in eliminating leukemic stem cells (LSCs), the cell of origin that initiates leukemia and drives relapse. A better understanding of the mechanisms of LSC chemotherapy evasion is therefore likely required for realization of a cure in AML. Directly studying the response of patient LSCs to induction therapy *in vivo* has the potential to identify novel chemoresistance pathways for the development of targeted therapies in combination with the current standard-of-care. This chapter will present an overview of both the normal and diseased hematopoietic systems, our understanding of LSC biology, and the past and current efforts of identifying and inhibiting chemoresistance pathways in AML.

NORMAL HEMATOPOIESIS

Hematopoiesis is the life-long process by which all cells of the blood system are replenished as they are lost or turned over. This is accomplished by hierarchical specification of multipotent, oligopotent, and lineage committed progenitors, all originating from a rare population of hematopoietic stem cells (HSCs) (Seita & Weissman, 2010).

Hematopoietic tissues

Hematopoiesis begins in humans as early as embryonic day 21 (E21) in the yolk sac blood islands, and continues through the entirety of fetal development as HSCs rapidly cycle and proliferate to establish the blood system (Pietras, Warr, & Passegué, 2011). HSCs in mice begin to colonize the aorta-gonad-mesonephros region from E8.5, the fetal liver from E11.5, and finally the bone marrow (BM) from E17.5, where they continue to expand through the first 3-weeks of postnatal life and then abruptly switch to a more quiescent state by 4-weeks (Bowie et al., 2006; Mikkola & Orkin, 2006). The major site of adult hematopoiesis in both mice in humans is the bone marrow although extramedullary hematopoiesis can also occur in the spleen and liver during times of stress, such as bone marrow failure or tissue injury (Johns & Christopher, 2012; Kiel & Morrison, 2008).

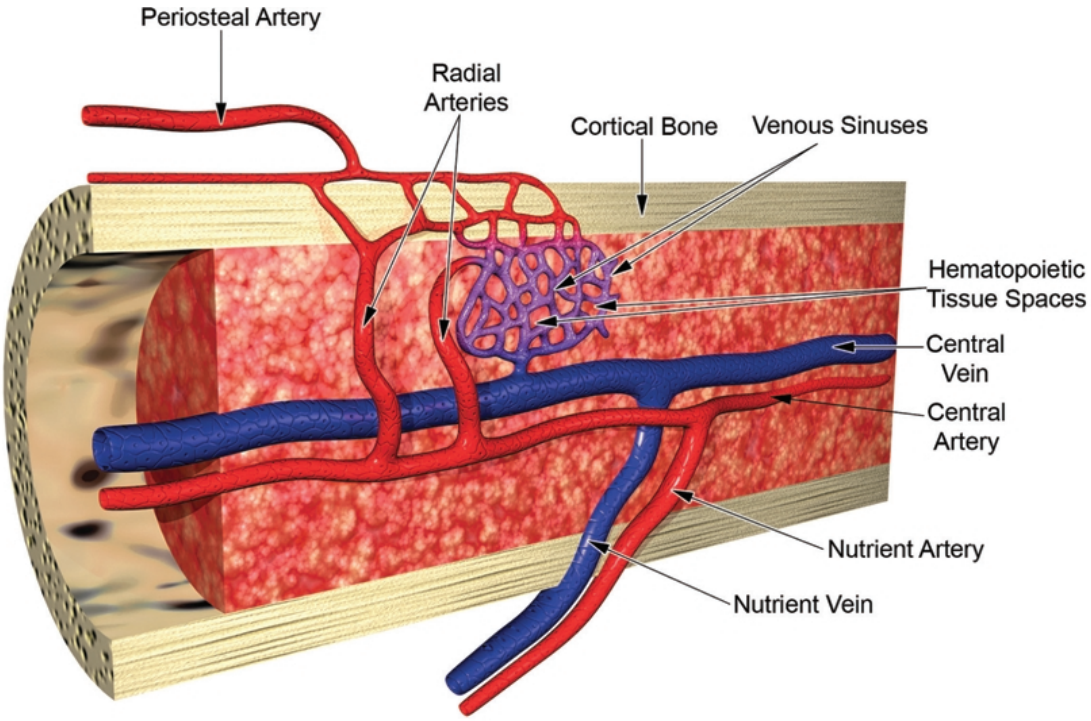
The majority of hematopoiesis and primary lymphogenesis occurs in the bone marrow of the axial and long bones. The bone marrow is located in the central bone cavity and consists of a multitude of cell types, areas of cellular development, and specialized biological functions. The endosteal surface of the bone marrow is inhabited by a heterogeneous mixture of cells including bone-synthesizing osteoblasts,

bone-resorbing osteoclasts, and mesenchymal progenitor cells (Kiel & Morrison, 2008). Arteries that carry oxygen, nutrients and growth factors into the bone marrow end in sinusoids before turning into the venous circulation. Various cells surround the sinusoids, including reticular cells, mesenchymal progenitors, megakaryocytes, monocytes, and endothelial cells. The marrow also consists of diverse hematopoietic tissue islands arranged in a deliberate fashion, including: Erythroblastic islands, granulopoietic foci, and areas of megakaryopoiesis adjacent to the sinus endothelium (Weiss & Geduldig, 1991). The structure of the bone marrow is more completely reviewed in: (Travlos, 2006) (**Figure 1-1**). Together, these various anatomical features orchestrate a complex interplay required for functionally healthy hematopoiesis.

Hematopoietic stem cells and lineage specification

HSCs reside in specialized microenvironments within the bone marrow, collectively referred to as the bone marrow niche. Both endosteal and perivascular regions have been shown to support HSCs, however the exact location of the HSC niche has remained elusive (Ehninger & Trumpp, 2011; Kiel & Morrison, 2008). It is through cell-cell, cell-extracellular matrix, and receptor-ligand interactions with the niche that HSCs are maintained and induced to respond to stimuli.

Despite their crucial role in blood regeneration, only 5% of adult HSCs actively divide under homeostatic conditions (Cheshier, Morrison, Liao, & Weissman, 1999; Pietras et al., 2011). The vast majority of HSCs are maintained in a dormant or quiescent state, ensuring their longevity and function, while leaving HSCs poised to re-enter the cell cycle when additional hematopoiesis is required



<u>Figure 1-1:</u> Bone marrow structure and its components

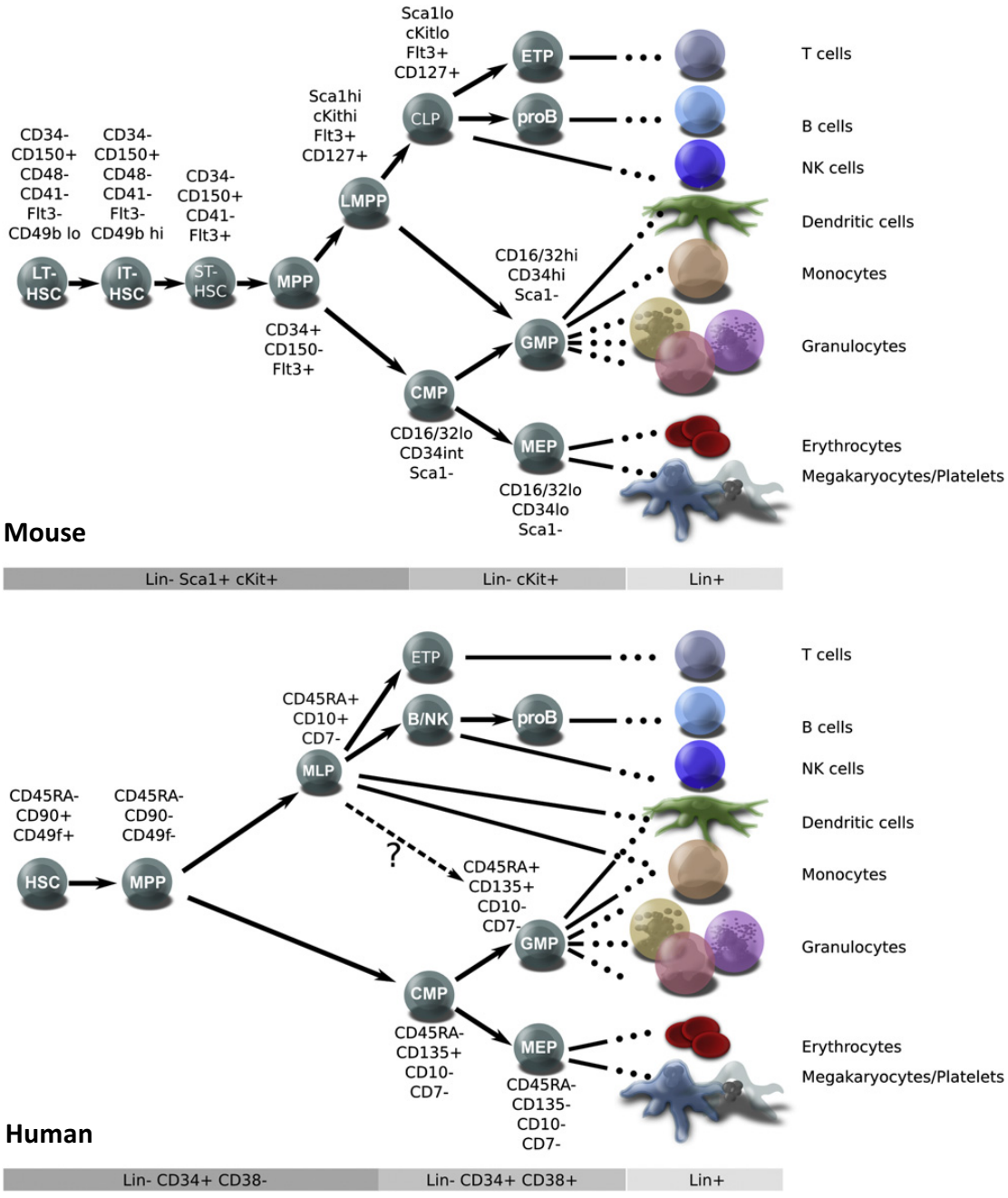
From: (Travlos, 2006)

(Kiel & Morrison, 2008; J. Li, 2011). HSC self-renewal and quiescence are highly associated with long-term reconstituting capacity and are critical for maintenance of the stem cell compartment (Blank, Karlsson, & Karlsson, 2008). Defects in regulation of either self-renewal or quiescence can lead to exhaustion of the HSC pool, resulting in hematopoietic failure (Ding & Morrison, 2013; Ding, Saunders, Enikolopov, & Morrison, 2012; L. Li & Bhatia, 2011; Qian et al., 2007). The decision to withdraw from the cell cycle, or undergo rounds of symmetric/asymmetric division is a highly regulated process involving both HSC-intrinsic and -extrinsic factors (Chotinantakul & Leeanansaksiri, 2012; J. Li, 2011; L. Li & Bhatia, 2011).

HSCs have the ability to give rise to all mature lineages of the blood, with as little as one HSC providing long-term hematopoietic reconstitution (Kiel et al., 2005). Through asymmetric division and symmetric differentiation HSCs may give rise to a hierarchy of progenitors that become progressively more lineage-restricted. Terminal differentiation results in production of mature blood cells, including erythrocytes, macrophages, granulocytes, megakaryocytes, and lymphocytes (**Figure 1-2**).

Molecular markers of hematopoietic lineages

The stem cell biology of the hematopoietic system is arguably one of the best characterized organ system under study. Over the past several decades increased understanding of phenotypic expression patterns of cell type-specific cell surface markers has allowed for refined prospective identification of many cells of the blood (Purton & Scadden, 2007). Lineage-specific markers for B cells ($B220^+$), T cells ($CD3^+/CD4^+/CD8^+$), granulocytes ($Mac\text{-}1^+Gr\text{-}1^+$), monocytes ($Mac\text{-}1^+Gr1^-$), megakaryocytes ($CD41^+$), and erythrocytes ($Ter119^+$) are well described. The



<u>Figure 1-2:</u> The hematopoietic hierarchy

Adapted from: (Doulatov, Notta, Laurenti, & Dick, 2012)

majority of immature stem and progenitor cells are defined as lineage-negative (Lin^-) and c-Kit^+. Within this population HSCs are additionally Sca-$1^+CD34^-CD150^+$, while multipotent progenitors are Sca-$1^+CD34^+CD150^{hi/low}$. Myelo-erythroid progenitors, including: common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP) are Lin^-c-Kit^+Sca-1^-. CMPs, GMPs, and MEPs are further defined by being $CD34^{int}CD16/32^-$, $CD34^+CD16/32^+$, or $CD34^-CD16/32^-$, respectively. The same strategy has been employed for the identification of human stem and progenitor cells. Cell surface markers for mouse and human are summarized in (**Figure 1-2**). The ability to isolate highly purified cell populations has facilitated detailed analysis of their transcriptional and epigenetic landscapes in recent years (Ivanova et al., 2002; Ji et al., 2010), which has broadened our understanding of molecular and biochemical pathways that underlie HSC function.

Indeed, much effort has been devoted to the identification of factors that influence HSC survival and self-renewal (Antonchuk, Sauvageau, & Humphries, 2002; Lessard & Sauvageau, 2003; Varnum-Finney et al., 2000). Genetic deletion of several cell cycle regulators and transcription factors, such as Rb, p53, p57, and Foxo1/3/4 show increased HSC cell cycle activity (Pietras et al., 2011). Several extrinsic factors have been shown to regulate HSC fate decisions, including angiopoietin-1 from bone-lining osteoblasts, CXCL12 and stem cell factor (SCF) secretion from endothelial and $lepr^+$ perivascular cells, as well as adherence interactions through integrin and N-cadherin (Ding & Morrison, 2013; Ding et al., 2012; J. Li, 2011). Administration of exogenous SCF and TPO cytokines, as well as various interleukins including: IL-3, IL-6, and IL-11, have been shown to promote both survival and proliferation of mouse HSCs *ex vivo* (Seita et al., 2007; Seita & Weissman, 2010).

Interestingly, the majority of transcription factors that are essential for hematopoiesis are often also linked to the promotion of leukemia. Such genes include: mixed lineage-leukemia gene (MLL), Runx1, TEL/ETV6, SCL/tal1, and LMO2, which constitute the majority of leukemia-associated translocations in patients (Orkin & Zon, 2008). Chimeric gene fusions can exert aberrant downstream effects on global transcription programs through target gene activation or repression and recruitment of alternative chromatin-modifying enzymes (Rosenbauer & Tenen, 2007). Several other genes involved in human HSC identification and lineage specification, including Flt3 and Dmnt3a, are also associated with poorer prognosis in patients with AML (Mclellan et al., 2010; Mizuki et al., 2000). Therefore, understanding of hematopoiesis is intrinsically linked to our understanding of leukemia. A more complete picture of HSC maintenance also has the added potential to facilitate their prolonged expansion *ex vivo* for clinical applications such as transfusable blood production and bone marrow transplantation.

ACUTE MYELOID LEUKEMIA

Over 250,000 adults are diagnosed with AML each year worldwide (Rowe & Tallman, 2010). AML can arise due to preexisting hematological malignancies or from prior therapy, however the majority of new AML cases develop *de novo* in previously healthy patients (De Kouchkovsky & Abdul-Hay, 2016; Sill, Olipitz, Zebisch, Schulz, & Wölfler, 2011). The disease is characterized by an uncontrolled expansion of immature myeloid cells in the bone marrow resulting in ineffective hematopoiesis and consequences of peripheral cytopenias. Despite recent advances in our understanding of the genetic origins of AML, treatment options remain limited and

outcomes are very poor (Roboz, 2011). Development of novel therapeutic approaches is therefore of great clinical importance.

Clinical progression

AML is defined by the abnormal proliferation and differentiation of a clonal population of myeloid cells, referred to as leukemic blasts. Like normal hematopoiesis, leukemic blasts are generated in a hierarchical manner beginning with a self-renewing population of leukemic stem cells (LSCs, $CD34^{+}CD38^{-}CD90^{-}$), resulting in distinct populations of leukemic progenitor (LPCs, $CD34^{+}CD38^{+}$) and leukemic blast ($CD34^{-}$) cells (Bonnet & Dick, 1997). However, unlike normal hematopoiesis where proliferation ceases and differentiation runs to completion, there is a block in development at the early myeloid stage, resulting in accumulation of immature cells with heightened proliferative capacity. These leukemic blasts exhibit varying, but incomplete, phenotypic characteristics indicative of abnormal development (Olsson, Bergh, Ehinger, & Gullberg, 1996).

The diagnosis of AML is established by the presence of greater than 20% blasts in the bone marrow or peripheral blood (Döhner et al., 2010). The myeloid origins of the disease are then further identified through immunophenotyping or myeloperoxidase activity to distinguish the disease from other types of neoplasms. Recent advances in our understanding of the underlying cytogenetic and molecular genetic abnormalities of AML have allowed for more rigorous classification into six major disease entities, with 11 subtypes depending upon presence of distinct chromosomal translocations (**Table 1-1**). The 2016 update to the WHO classification of AML is further reviewed here: (Arber et al., 2016).

Table 1-1: WHO classification of AML

Table 1. WHO classification of AML and related neoplasms

Types	*Genetic abnormalites*
AML with recurrent genetic abnormalities	AML with t(8:21)(q22;q22); RUNX1-RUNX1T1
	AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
	APL with PML-RARA
	AML with t(9;11)(p21.3;q23.3); MLLT3-KMT2A
	ML with t(6;9)(p23;q34.1); DEK-NUP214
	AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
	AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); RBM15-MKL1
	AML with BCR-ABL1 (provisional entity)
	AML with mutated NPM1
	AML with biallelic mutations of CEBPA
	AML with mutated RUNX1 (provisional entity)
AML with myelodysplasia-related changes	
Therapy-related myeloid neoplasms	
	AML with minimal differentiation
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukemia
	Acute monoblastic/monocytic leukemia
	Acute erythroid leukemia
	Pure erythroid leukemia
	Acute megakaryoblastic leukemia
	Acute basophilic leukemia
	Acute panmyelosis with myelofibrosis
Myeloid sarcoma	
Myeloid proliferations related to Down syndrome	Transient abnormal myelopoiesis
	ML associated with Down syndrome

Abbreviations: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ML, myeloid leukemia; WHO, World Health Organization.

Adapted from: (De Kouchkovsky & Abdul-Hay, 2016)

Clinical manifestations of AML relate to peripheral cytopenias, including leukocytosis, anemia, and thrombocytopenia, which are indicative of underlying bone marrow failure. Fever, fatigue, bruising, weight loss, and issues relating to wound healing are also common. If left untreated, patients succumb to the disease within several months following diagnosis due to complications of infection or bleeding (De Kouchkovsky & Abdul-Hay, 2016).

Impact on hematopoiesis

In many ways, the aggressiveness of AML stems from an inability to sustain normal hematopoiesis. As malignant cells expand, they outcompete healthy cells for nutrients, space in the BM and periphery, and otherwise inflict deleterious changes to the BM niche. HSCs and LSCs share similar phenotypic markers, including CD34, CD38, CD71, and HLA-DR (Jordan, 2002) and are also dependent upon many of the same cytokines for growth, including IL-3, IL-6, and SCF. Gene expression changes in blasts also put them in direct competition for resources with more differentiated normal hematopoietic progeny.

Thrombopoietin (TPO), an important regulator of megakaryopoiesis and platelet production, also regulates maintenance of HSC self-renewal and quiescence (de Sauvage et al., 1996; Kimura, Roberts, Metcalf, & Alexander, 1998). High expression of the TPO receptor MPL on leukemic blasts predicts neutropenia and thrombocytopenia in AML patients, and has been shown to deplete TPO in cell culture and in mouse models (Rauch et al., 2016).

Hyperproliferation of AML blasts also has significant detrimental effects on bone marrow composition and niche maintenance. Bone marrow stroma from AML

patients have been shown to be unable to support normal blood cell formation, with two thirds of AML patient cases demonstrating abnormally low levels of colony-stimulating factor secretion from bone marrow monocytes and macrophages (Dührsen & Hossfeld, 1996; Peter & Greenberg, 1978). AML blasts also secrete higher levels of matrix metalloproteinases, specifically MPP-2, which may lead to changes in BM extracellular matrix composition (Ries, Loher, Zang, Ismair, & Petrides, 1999). Increased hypoxia in the leukemic bone marrow also alters expression of many pro-survival factors in AML blasts, including HIF-1a and FLT3, representing a potential positive feedback loop between altered BM microenvironment and enhanced leukemic cell survival (Benito, Zeng, Konopleva, & Wilson, 2013; Drolle et al., 2014; Sironi et al., 2015). Additionally, it was shown that the tumor microenvironment sequesters normal $CD34^+$ cells, resulting in a decline in their frequency, likely due to leukemic cell SCF secretion (Colmone et al., 2008).

These perturbations to the BM are exacerbated in elderly patients with AML. The aged bone marrow is typified by onset of anemia, decreased competence of the adaptive immune system, and expansion of myeloid cells, potentially due to elevated levels of the pro-inflammatory CC-chemokine ligand (CCL5) secreted by the BM microenvironment. Aged HSCs also demonstrate cell-intrinsic changes that alters their self-renewal, homing, and differentiation capacities, as demonstrated by elevated HSC frequencies, niche preferences in transplantation, and lymphoid progenitor output (Geiger, de Haan, & Florian, 2013). Together, the complications of AML are a result of competition between leukemic blasts proliferation and normal hematopoiesis inside an increasingly compromised BM niche, which is further exacerbated by inherent hematopoietic deficiencies in the elderly.

Genetic and epigenetic alterations

While advanced age is a major adverse prognostic factor (Appelbaum et al., 2015), there are also numerous genetic and epigenetic abnormalities that are predictive of patient outcome. Genetic mutations are identified in more than 97% of AML cases (J. P. Patel et al., 2012). Younger adult patients are commonly grouped into favorable, intermediate, or adverse risk based cytogenetics (Grimwade et al., 1998). A complex karyotype with three or more chromosomal abnormalities is associated with very poor outcome (Mrózek, 2008), which occurs in 10-12% of patients. It is unclear how the interplay between multiple cytogenetic changes relates to AML pathogenesis, however the non-random pattern of these mutations is suggestive of a stepwise acquisition of genetic aberrations (Dash & Gilliland, 2001).

Indeed, the karyotype of leukemic cells has been shown to be one of the strongest prognostic factors for response to induction therapy and survival (Mrózek, Heerema, & Bloomfield, 2004). More than 100 different chromosomal translocations have been cloned in AML (Gilliland & Tallman, 2002). Several gene-fusions are highly associated with development of AML, including: RUNX1-RUNX1T1, CBFB-MYH11, PML-RARA, MLLT3-KMT2A, DEK-NUP214, GATA2, MECOM, RBM15-MKL1. These gene fusions result in enforced expression of hematopoietic transcription factors. The aggressiveness of AML in elderly patients is, in part, attributable to increased incidence of adverse vs favorable chromosomal abnormalities in this patient group (Leith et al., 1997).

Numerous models have been proposed to explain acquisition of clonal genetic defects in AML. One of the most widely cited mechanisms, the two-hit model, postulates that the critical events in AML pathogenesis are a mutation in a

hematopoietic transcription factor combined with an activating mutation in a kinase. In this model, class I mutations activate pro-proliferative pathways, while class II mutations impair normal hematopoietic differentiation (De Kouchkovsky & Abdul-Hay, 2016). Support of this comes from studies describing the insufficiency of single transgenes for HRX-ENL, AML/MDS/EVI, and E2A-Pbx1, among others, in inducing acute leukemia (Cuenco & Ren, 2001; Lavau, Szilvassy, Slany, & Cleary, 1997; Thorsteinsdottir et al., 1999). This model helps explain how the sheer number of mutational combinations observed in AML can result in grossly similar disease.

Examples of class I mutations that promote proliferation include FLT3 ITD, K/NRAS, TP53, and c-Kit are found in roughly 28%, 12%, 8%, and 4% of cases, respectively (Voigt & Reinberg, 2013a). Enhanced phosphorylation of STAT3 is also seen in up to 50% of AML cases, typically conferring a worse prognosis (Ghoshal, Baumann, & Wetzler, 2008). Class II mutations include NPM1 and CEBPA, which are found in roughly 27% and 6% of cases, respectively (Kihara et al., 2014; Voigt & Reinberg, 2013b). A third class of genes that affect epigenetic regulation and can simultaneously influence proliferation and differentiation have also recently emerged, including DNMT3A, TET2, and IDH-1/2 (Challen et al., 2012; J. P. Patel et al., 2012). Mutations in these genes are found in more than 40% of AML cases.

Studies over the past several decades have shown that AML is a highly heterogeneous disease, with interpatient variations significantly influencing prognosis and treatment choice. While our understanding of the mutational drivers of AML have evolved significantly, our grasp of how these genes dictate response to therapy is still relatively naive. Characterization of gene expression changes in response to therapy in specific AML subtypes represents a logical next step to expanding our understanding of AML biology and development of new therapeutics.

Treatment

The standard induction therapy regimen is comprised of a combination of seven days of the nucleoside analog cytarabine (Ara-C) and three days of an anthracycline such as daunorubicin (DNR) (Ogbomo, Michaelis, & Klassert, 2008; Quigley et al., 1980; Roboz, 2011; Rowe & Tallman, 2010). 7+3 induction therapy has remained the standard of care for over four decades and complete remission (CR), defined as < 5% BM blasts, is achieved in 60% to 80% of young adults, which is unrivaled by any other current treatment option (Döhner et al., 2010) (**Table 1-2**). However without additional therapeutic interventions patients typically relapse, resulting in disease resistant to current therapies (Burnett et al., 2009; Cassileth et al., 1992).

Postremission strategies depend upon age, cytogenetics, and molecular genetic risk. Postremission consolidation and maintenance in young patients and patients with favorable- and intermediate-risk cytogenetics typically consists of high-dose Ara-C (HiDAC) treatment followed by autologous or allogeneic hematopoietic stem cell transplantation (HSCT). 5-year remission rates are 65% in favorable-risk groups (Juliusson et al., 2014). In patients with adverse-risk AML outcomes are much worse, even with current consolidation methods (Grimwade et al., 1998). The treatment recommendation for this group is an allogeneic HSCT from a matched related donor, with survival of 44% versus 15% of patients receiving a single cycle of HiDAC (Slovak et al., 2015). Management of older patients (>60 years of age) is often more complicated. Increasing age is associated with poor performance status and comorbidities that can affect tolerance to induction therapy. Elderly patients are also segregated into classes of increasing risk, which are highly correlated with their

Table 1-2: Treatment options for AML

Anthracyclines (Antitumor Antibiotics)

- daunorubicin (Cerubidine®)
- doxorubicin (Adriamycin®)
- idarubicin (Idamycin®)
- mitoxantrone (Novantrone®)

Antimetabolites

- cladribine (2-CdA; Leustatin®)
- clofarabine (Clolar®)
- cytarabine (cytosine arabinoside, ara-C; Cytosar-U®)
- fludarabine (Fludara®)
- hydroxyurea (Hydrea®)
- methotrexate
- 6-mercaptopurine (Purinethol®)
- 6-thioguanine (Thioguanine Tabloid®)

Topoisomerase Inhibitors

- etoposide (VP-16; VePesid®, Etopophos®)
- topotecan (Hycamtin®)

DNA Damaging (Alkylating) Agents

- cyclophosphamide (Cytoxan®)
- carboplatin (Paraplatin®)
- temozolomide (Temodar®)

Cell-Maturing Agents

- all-*trans* retinoic acid (ATRA, tretinoin; Vesanoid®)
- arsenic trioxide (Trisenox®)

Hypomethylating Agents

- azacitidine (Vidaza®)
- decitabine (Dacogen®)

Table 3. | This table lists some of the standard drugs and some of the drugs under study in clinical trials to treat AML patients. Various approaches to AML treatment are undergoing study in clinical trials. A patient may be treated with drugs that are not listed in this table and still be receiving appropriate and effective treatment. For a description of standard chemotherapy combinations, see page 15. It is essential to seek treatment in a center where doctors are experienced in the care of patients with acute leukemia.

From: (Karp, 2011)

cytogenetics. In elderly patients with adverse cytogenetics, CR rates are 30% or less, and overall survival (OS) is less than 5% (Juliusson et al., 2014). There is also heightened therapy-related death and chemotherapy resistance in this group (Grimwade et al., 2001).

A deeper understanding of the genetic basis of AML in recent years has led to the development of several molecularly-targeted therapies. Cytotoxic agents linked to monoclonal antibodies against the myeloid-associated antigen CD33 (gemtuzumab ozogamicin), a cell surface marker highly expressed on leukemic blasts, was shown to produce remission in 15% to 35% of older patients, and up to 91% in younger patients in first relapse (Kell et al., 2003). FLT3 inhibitors (midostaurin, lestaurtinib, sunitinib) and demethylating agents (azacitidine) are also being developed for the treatment of AML, the later demonstrating 2-year OS of 50% in older patients (Fenaux et al., 2010; Wander, Levis, & Fathi, 2014). Widespread adoption of these targeted therapies depends upon their demonstrated therapeutic index, as well as CR and OS rates compared to, or in combination with, induction chemotherapy.

Despite progress in our management of AML, there are still considerable questions relating to the appropriate number of treatment cycles, dosage and schedule, and combination of HiDAC with other agents. The influence of discrete cytogenetic and molecular genetic abnormalities on treatment choice is still an area of active research. Greater understanding of the genetic and epigenetic underpinnings of AML have led to the identification and pharmacologic development against aberrant proteins in acute leukemia. But with the exception of retinoic acid and arsenic trioxide for acute promylocytic leukemia (APL) (S.-J. Chen & Zhou, 2012), these therapies have not significantly improved treatment responses alone, likely reflecting the influence of multiple genetic lesions that drive AML.

Issues with treatment

There are many hurdles that must be overcome to improve the current standard of care for AML, including but not limited to: Non-specificity of induction therapy between leukemic and normal cells, the neurological and cardiotoxicity of Ara-C and anthracyclines, low tolerance to chemotherapy in the elderly, and the inability for current therapies to directly target LSCs.

As with most chemotherapeutic compounds, induction therapy elicits its effect through non-specific targeting of rapidly dividing cells. Ara-C, a deoxycytidine analog, interferes with the synthesis of DNA through its incorporation into actively replicating DNA strands during S phase of the cell cycle. Ara-C also inhibits DNA and RNA polymerase and nucleotide reductase enzymes required for DNA synthesis (Momparler, 2013). Anthracyclines such as daunorubicin and doxorubicin inhibit rapidly dividing cells by intercalating between base pairs in DNA and RNA, as well as inhibition of topoisomerase II. Despite the ability of these compounds to effectively kill leukemic blasts in the periphery and BM, common, sometimes severe side effects also occur, including: Myelosuppression, rash, gastrointestinal toxicity, mucositis, and infection (Woelich et al., 2017). Anthracyclines also exhibit cumulative cardiac toxicity, which limits their use clinically. While occurrence of these side effects are often outweighed by the potential benefits of therapy in younger patients, treatment-related death (defined as death within four weeks of therapy initialization) occurs in roughly 5% of patients, with significantly worse tolerance, especially to high-dose chemotherapy, in the elderly (Döhner et al., 2010; Marceau et al., 2014).

As a percentage of total causes of death in AML, the risk of mortality due to therapy itself pales in comparison to the number of patients that die from relapsed

disease. Indeed, relapse continues to be the leading cause of death in patients with AML, regardless of cytogenetic classification and risk stratification (Ravandi & Estrov, 2006). Even in patients who achieve CR, relapse often occurs months or years later. Given their quiescent nature, high expression of ATP-associated transporters, and resistance to apoptotic stimuli, it is widely believed that LSCs are able to escape induction therapy and drive reoccurrence of AML (Dean, Fojo, & Bates, 2005; Guan, Gerhard, & Hogge, 2003; Konopleva et al., 2002). It is therefore imperative that we gain a better understanding of leukemic cell chemotherapy evasion, particularly in respect to LSCs, for the development of additional therapeutic approaches for complete eradication of the disease.

LEUKEMIC STEM CELLS

The clonal nature of AML suggests it arises from a single cell-of-origin. Similar to HSCs, LSCs are thought to lie at the root of clonal growth and therefore be the cell responsible for perpetuation of leukemia. LSCs share many phenotypic and functional characteristics with HSCs, including: Expression of similar cell surface markers, ability to self-renew, cell cycle quiescence, extensive proliferative capacity, and relative resistance to cytotoxic stress (Jordan, 2002). The presence of residual LSCs following induction therapy are thought to be the primary driver of relapse. The challenge of therapeutically targeting LSCs therefore lies in understanding the differences of LSCs not only from the bulk leukemic population, but also from normal HSCs and progenitor cells.

Identification

The idea that a small population of stem cells, with the ability to self-renew and assert homeostatic control for the maintenance and propagation of cancer, particularly in the context of leukemia, is not new (Fialkow, Gartler, & Yoshida, 1967). In the past several decades, many of the same techniques that were used for phenotypic and functional elucidation of normal hematopoiesis were also employed for the identification of LSCs, including prospective isolation based on defined phenotypic markers and leukemia initiating studies in long-term transplantation systems. Leukemia is now one of the best characterized systems for our understanding of cancer stem cells (J. C. Y. Wang & Dick, 2005).

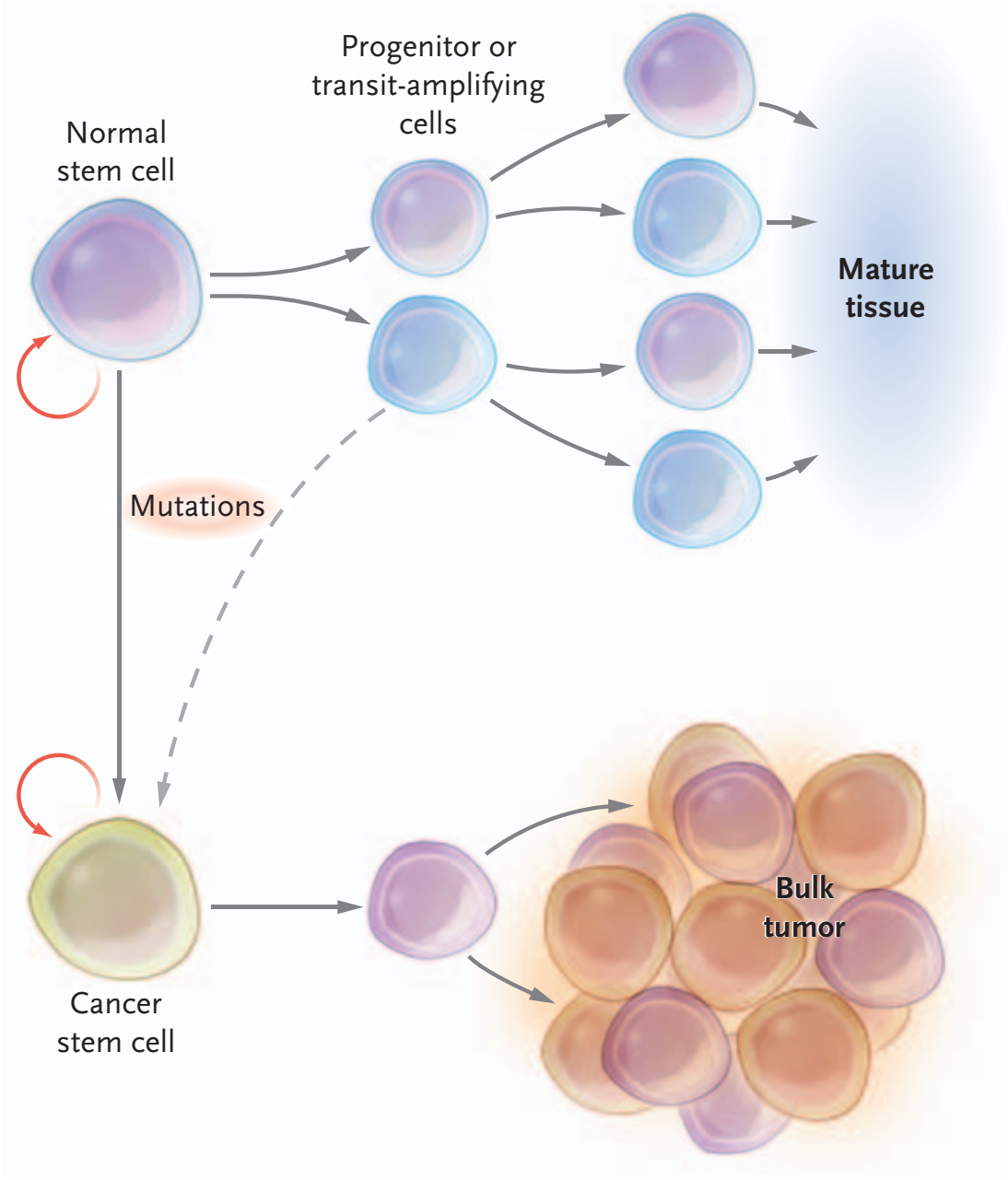
LSCs comprise anywhere from 0.1% to 1% of leukemic cells as assessed by leukemia initiating capacity of blasts injected into severe combined immunodeficient mice (Lapidot et al., 1994). The marker expression profile of LSCs is minimally defined as $CD34^{+}CD38^{-}CD90^{-}$ with additional enrichment for $CD117^{-}$ (c-Kit) and $CD123^{+}$ (IL-3R) expression (Blair, Hogge, Ailles, Lansdorp, & Sutherland, 1997; Bonnet & Dick, 1997; Jordan et al., 2000). Excitingly, high LSC expression of certain self-renewal pathways such as Wnt/ β-catenin, Notch, and Hedgehog (Dick, 2003; Horton & Huntly, 2012), cell surface antigens such as CLL-1, CD44, CD96, and CD47 (Jordan et al., 2000; Majeti et al., 2009), SRC family kinases (Santos et al., 2014), and anti-apoptotic molecules such as Bcl-2 (Lagadinou et al., 2013), have been identified in recent years. This may offer unique therapeutic targets to eliminate LSCs while maintaining the normal HSC pool, an endeavor that has already produced several drug candidates (Feuring-Buske, Frankel, Alexander, Gerhard, & Hogge, 2002; Hosen et al., 2007; Santos et al., 2014). Therefore, additional understanding of

the mutagenic and gene expression events that occur in LSCs during malignant transformation and response to induction therapy will likely drive clinical development in coming years.

Malignant transformation of LSCs

LSC malignant transformation is thought to be a result of mutations in primitive hematopoietic cells (**Figure 1-3**). LSC cell surface expression patterns, self-renewal capacity, and ability to drive hierarchical, albeit incomplete, hematopoietic specification suggests that LSCs share many similarities to HSPCs. The question is then, do LSCs arise directly from HSCs that have accumulated mutations such as loss of programmed cell death, or restricted progenitors that have gained an enhanced capacity to self-renew? Due to the heterogeneity of LSCs it has been unclear for many years whether AML results directly from mutations in HSCs or other more mature precursor cells (Passegué, Jamieson, Ailles, & Weissman, 2003). However, the development of refined LSC detection methods and more effective immunodeficient xenograft models has shown that, at least in some cases, LSCs are present in multiple phenotypic compartments (Pollyea, Gutman, Gore, Smith, & Jordan, 2014).

There are several categories of mutagenic events that must occur for malignant transformation in AML, including: Increased self-renewal, impaired differentiation, and increased cell survival/proliferation (Reya, Morrison, Clarke, & Weissman, 2001). Mutations in genes that impart increased self-renewal, such as stabilization of β-catenin, or enforced signaling in pathways that promote progenitor self-renewal, such as Hox genes, Notch, and Shh have been described (Lawrence, Sauvageau, Humphries, & Largman, 1996; Niemann et al., 2003). Gene fusions that directly



<u>Figure 1-3:</u> LSC transformation in AML

From: (Jordan, Guzman, & Noble, 2006)

impair hematopoietic differentiation, typically consisting of a transcription factor, such as AML1 or RARa, and a variable fusion partner, which may control cell survival and apoptosis such as PML, are also hallmarks of AML and are detectable in LSCs (Alcalay et al., 2001). Gain-of-function mutations in cell death antagonists, such as Bcl-2, Bcl-xl, and Mcl-1 have also been linked to leukemia. Most human AMLs express elevated levels of Bcl-2, and the AML1-ETO fusion has been shown to upregulate expression of Bcl-2 through binding its promoter elements (Delia et al., 1992; Klampfer, Zhang, Zelenetz, Uchida, & Nimer, 1996). Like most cancers, acquisition of these mutations in AML is thought to occur in a stepwise manner, resulting in an increasingly transformed clonal population of leukemic cells (Hanahan & Weinberg, 2000).

Role in AML pathogenesis and relapse

Regardless of whether the driver mutations are accumulated in a cell that was once an HSC or more committed progenitor, the transformed LSC has the ability to potentiate primary leukemic outgrowth, as well as relapse following therapy. Studies in recent years have shown significant inter-patient variability in LSC marker expression and mutational status, likely arising as a consequence of difference in disease pathogenesis (Eppert et al., 2011; Sarry et al., 2011). Transplanted LSCs give rise to secondary AML with a similar phenotype observed in the original patient, which speaks to the clonal nature of AML (Ho et al., 2016). Patients harboring a higher LSC frequency or a more prevalent stem cell phenotype at diagnosis also exhibit significantly poorer outcomes than patients with fewer LSCs or a less prevalent stem cell phenotype (Gentles, Plevritis, & Page, 2012; Ran et al., 2012; Van

Rhenen et al., 2005). Enrichment for an LSC gene expression signature results in significantly worse overall, event-free, and relapse-free survival in patients regardless of karyotype status (Gentles et al., 2012; Ng et al., 2016). Therefore, a 'stemness' signature is thought to be an independent prognostic indicator of AML aggressiveness and patient response to treatment. The relatively high LSC frequency (25-30% of all myeloid cells) may also help explain the particularly aggressive nature of the MLL-AF9 leukemic mouse model (Somervaille & Cleary, 2006).

Persistence of LSCs following therapy is also a primary driver of relapse. To appreciate the role of LSCs in this process, it is helpful to understand clinical classification of response to therapy. Induction therapy results in varying amounts of bulk tumor reduction, classified as complete remission (CR, < 5% BM blasts), complete remission with incomplete recovery (CRi, CR except residual neutropenia or thrombocytopenia), and molecular complete remission (CRm, reversion to normal karyotype) (Cheson et al., 2003; Döhner et al., 2010). A summary of response criteria in AML are depicted in **Table 1-3**. The heterogeneity in patient response is currently not well understood, however it is likely due to a combination of driver mutations, level of differentiation within the tumor, and patient-specific comorbidities (Estey, 2009). While our ability to detect residual leukemia at the molecular level by flow cytometry and PCR has improved consolidation and supportive care following induction therapy, the limit of detection is still a major hurdle in ensuring the complete elimination of leukemia (Grimwade & Freeman, 2016). Therefore, although a patient may be classified as CRm, many still harbor minimal residual disease (MRD), comprised of a population of undetectable cells that have evaded therapy.

Relapsed leukemia arises if this residual population contains leukemic cells that are disease-sustaining, with length of remission predicted by residual LSC self-

Table 1-3: Response criteria in AML

Category	Definition
Complete remission (CR)*	Bone marrow blasts < 5%; absence of blasts with Auer rods; absence of extramedullary disease; absolute neutrophil count > 1.0 × 10^9/L (1000/μL); platelet count > 100 × 10^9/L (100 000/μL); independence of red cell transfusions
CR with incomplete recovery (CRi)†	All CR criteria except for residual neutropenia (< 1.0 × 10^9/L [1000/μL]) or thrombocytopenia (< 100 × 10^9/L [100 000/μL])
Morphologic leukemia-free state‡	Bone marrow blasts < 5%; absence of blasts with Auer rods; absence of extramedullary disease; no hematologic recovery required
Partial remission (PR)	Relevant in the setting of phase 1 and 2 clinical trials only; all hematologic criteria of CR; decrease of bone marrow blast percentage to 5% to 25%; and decrease of pretreatment bone marrow blast percentage by at least 50%
Cytogenetic CR (CRc)§	Reversion to a normal karyotype at the time of morphologic CR (or CRi) in cases with an abnormal karyotype at the time of diagnosis; based on the evaluation of 20 metaphase cells from bone marrow
Molecular CR (CRm)‖	No standard definition; depends on molecular target
Treatment failure	
Resistant disease (RD)	Failure to achieve CR or CRi (general practice; phase 2/3 trials), or failure to achieve CR, CRi, or PR (phase 1 trials); only includes patients surviving ≥ 7 days following completion of initial treatment, with evidence of persistent leukemia by blood and/or bone marrow examination
Death in aplasia	Deaths occurring ≥ 7 days following completion of initial treatment while cytopenic; with an aplastic or hypoplastic bone marrow obtained within 7 days of death, without evidence of persistent leukemia
Death from indeterminate cause	Deaths occurring before completion of therapy, or < 7 days following its completion; or deaths occurring ≥ 7 days following completion of initial therapy with no blasts in the blood, but no bone marrow examination available
Relapse¶	Bone marrow blasts ≥ 5%; or reappearance of blasts in the blood; or development of extramedullary disease

From: (Döhner et al., 2010)

renewal and proliferation activity (Stiehl, Baran, Ho, & Marciniak-Czochra, 2015). The MRD population typically resembles the disease at diagnosis, suggesting that MRD contains LSCs (Van Rhenen et al., 2007). A higher percentage of LSCs in complete remission following induction therapy strongly correlates with shorter patient survival, which can be further stratified based on frequencies of MRD (Terwijn et al., 2014). This, combined with early studies demonstrating the ability of a single transplanted LSC to initiate leukemia in mice (Somervaille & Cleary, 2006), point to LSCs as the cell-of-origin not only for leukemia initiation, but also for relapse.

Together, these findings highlight therapeutic targeting of LSCs as the next big hurdle in AML treatment. LSCs have been shown to perpetuate the clonal progression of AML, with initial mutations likely driving malignant transformation at the level of HSPCs. The intrinsic resistance and stem cell properties of LSCs renders them uniquely suited to evade induction therapy and drive relapse. More complete elimination of LSCs will likely come from improved detection through LSC-specific biomarkers in MRD combined with novel therapies that simultaneously capitalize upon LSC-specific dependencies while avoiding normal HSPCs. Better understanding of how to apply the unique characteristics of LSCs as targets for therapeutic advantage is therefore required.

CHEMORESISTANCE MECHANISMS IN AML

There are multiple examples of why LSCs may be more resistant to therapy than the bulk leukemia. The quiescent nature of the LSC may render these cells impervious to the genotoxic stress of chemotherapy, which primarily targets dividing cells. Enhanced expression of multiple drug efflux pumps, including ABCB1,

ABCC1, and ABCG2, have also been postulated as mediators of LSC chemoresistance (Dean et al., 2005). Elevated levels of various anti-apoptotic proteins, such as Bcl-2, Bcl-xL, and Mcl-1 have been associated with leukemogenesis and poor response to therapy (Y.-H. Wang & Scadden, 2015). Several of these processes are depicted in **Figure 1-4**. Notably, the role of additional biological activities in chemoresistance, including: Inflammation, lipid metabolism, and epigenetic modification, has been more greatly appreciated in recent years (de Visser & Jonkers, 2009; Guryanova et al., 2016; H. Ye et al., 2016). Expanded understanding of these processes in relation to chemoresistance and LSC biology has the promise to uncover additional targets for therapeutic development.

Drug efflux

Transmembrane drug transporters where one of the first described mechanisms of chemotherapy evasion (Juliano & Ling, 1976). Despite the clear role of ABC-family transporters in mediating multi-drug resistance (MDR) in leukemic cells both *in vitro* and *in vivo*, numerous agents developed to inhibit ATP-binding cassette transporters, including P-glycoprotein (P-gp) and MRP1 (ABCC1), have found limited clinical efficacy (Callaghan, Luk, & Bebawy, 2014). First and second generation P-gp inhibitors were abandoned due to poor potency and off-target effects that caused severe toxicities, with third generation P-gp inhibitors also demonstrating poor pharmacokinetic profiles in combination with induction chemotherapy (Gottesman et al., 2002). Additionally, both LSCs and HSCs have been shown to express heightened levels of drug efflux pumps, rendering them equally as susceptible to these inhibitors (Dean et al., 2005). Therefore, while it is likely that transporters

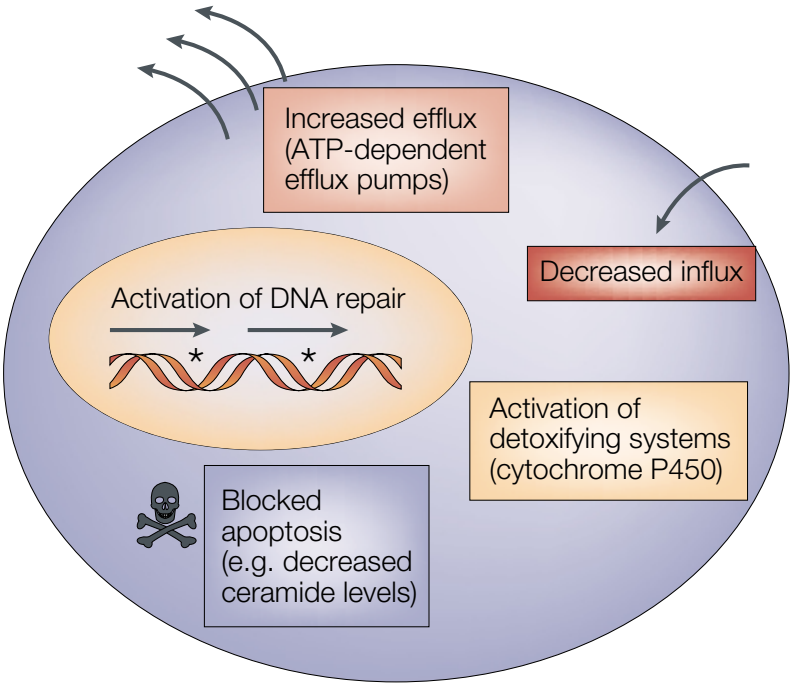


Figure 1-4: Examples of chemoresistance mechanisms

From: (Gottesman, Fojo, & Bates, 2002)

mediating drug efflux are important arbitrators of chemoresistance, the clinical utility of inhibiting these proteins is questionable.

It may be possible to circumvent the MDR and dose limiting toxicities of chemotherapeutic compounds through drug encapsulation in liposomes or other polymers. Indeed, recent positive Phase III trial results for CPX-351, a liposomal formulation of Ara-C and DNR, are very exciting for the treatment of patients with poor drug tolerance (Lancet et al., 2016). This approach has the potential to increase local concentrations of Ara-C and anthracyclines, which would eliminate the need to inhibit their efflux from the cell. Additional carriers that incorporate a packaged high-dose of an anthracycline with an siRNA directed against the *mdr1* gene have shown promise (Susa et al., 2010). Additional advances in delivery and stabilization of induction therapy are likely to continue in coming years, however the non-specificity of both induction therapy and drug efflux inhibitors underscore the importance of identifying mechanisms of drug resistance that are unique to leukemic cells.

Cell cycle and self-renewal

Quiescence is a characteristic inherent in the biology of stem cells (Pietras et al., 2011). The vast majority of HSCs are maintained in dormancy, yet are poised to re-enter the cell cycle when additional hematopoiesis is required (Kiel & Morrison, 2008; J. Li, 2011). If LSCs originate from HSPCs, it is easily extrapolated that they must also be quiescent as well. Indeed, in one study as many as 96% of observed LSCs ($CD34^+CD38^-CD123^+$) were in G_0 phase of the cell cycle (Guzman et al., 2001). This may represent an innate process by which LSCs evade chemotherapy.

Several cell cycle regulators have been shown to be induced with anthracycline

treatment (Laurent, Jaffrézou, & Jaffre, 2001). Doxorubicin treatment induces p53 activation, leading to the induction of WAF1/CIP1 p21 gene expression, a strong inhibitor of cyclin-dependent kinases responsible for G1 to S transition (El-Deiry et al., 1993). High levels of WAF1/CIP1 protein are also associated with chemoresistance in AML, which is reversed with MEK inhibition (Milella et al., 2001).

LSCs that do divide have been shown to possess a higher self-renewal capacity than normal SCID-repopulating cells (Bonnet & Dick, 1997). Studies in Bmi-1 knockout mice demonstrate that presence of Bmi-1 regulates the serial transplantability of Hoxa9/Meis1 driven AML (Lessard & Sauvageau, 2003). However, Bmi-1 and Hox family genes are important for both LSC and HSC self-renewal, limiting their clinical potential (Warner, Wang, Hope, Jin, & Dick, 2004). Additional self-renewal associated signaling pathways include: Hedgehog (Hh), canonical Wnt, FoxO, and Notch; all of which have defined roles in embryonic development and have in the past decade been tied to leukemic development and chemoresistance (Heidel, Mar, & Armstrong, 2011). Furthermore, increased expression of Musashi-2 (Msi2), a cell fate determinant that directs symmetric division, was associated with aggressive disease and immature phenotype in human AML (Ito et al., 2010; Kharas et al., 2010). These studies suggest that the enhanced self-renewal capacity of LSCs may be a determinant of ability to evade therapy and drive relapse.

Several inhibitors of LSC self-renewal pathways are currently under development for leukemia. Gamma-secretase inhibitors MK-0752 and PF03084014 are being tested for the treatment of T-ALL (NIH, n.d.). Other Notch pathway inhibitors, including the Delta-like ligand 4 (DLL4) antibody therapy, have been

shown to reduce stem cell frequency in solid tumors (Hoey et al., 2009). Hh inhibition with cyclopamine slowed disease onset and resulted in decreased colony forming units *in vitro* in combination with BCR-ABL inhibition in chronic myeloid leukemia (CML) (Dierks et al., 2008). Interestingly, the MEK inhibitors PD98059 and PD184352 demonstrated preferential effects on leukemic cell clonogenicity with minimal effects on normal hematopoietic progenitor cells, potentially through modulation of both CDK inhibitors and anti-apoptotic proteins (Milella et al., 2001). This suggests that there may be some selectivity when it comes to inhibiting LSC self-renewal, especially with pathways such as MAPK that are constitutively activated in many AML cases (Towatari et al., 1997).

Therapies targeting the cell cycle or promoting differentiation of LSCs can be implemented in several ways. Methods of pushing LSCs out of quiescence or promoting their differentiation could precede induction therapy to render them more sensitive to genotoxic stress. Alternatively, drugs that maintain LSCs in a quiescent state could be given chronically following induction therapy to suppress relapse and extend length of remission. Several examples of potential therapy sequencing are depicted in **Figure 1-5**. Importantly, these approaches are not mutually exclusive, with a combination of LSC-targeted therapies at different times during the treatment cycle potentially resulting in the best response.

Apoptotic regulators

For most anticancer therapies, including chemotherapy, the major mechanism of action is the activation of apoptosis (Y.-H. Wang & Scadden, 2015). Evasion of apoptotic stimuli is a hallmark of cancer, with LSCs thought to be less sensitive to

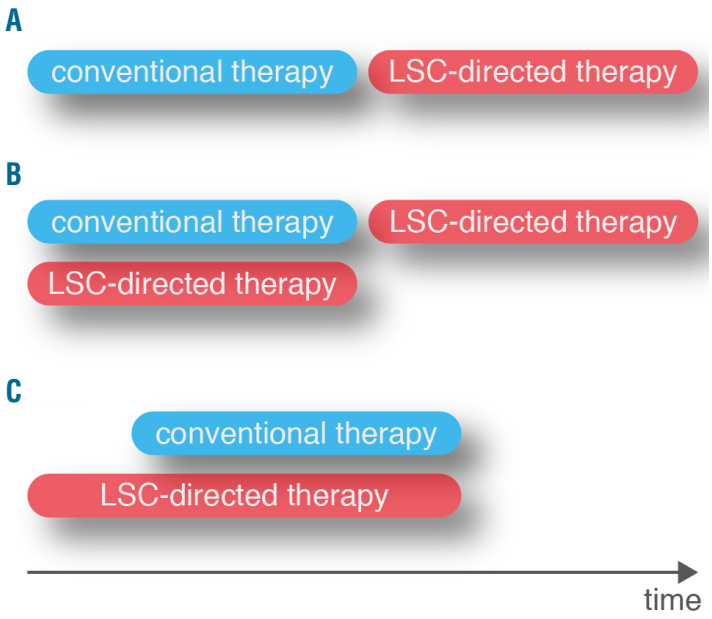


Figure 1-5: **Therapy sequencing in combinatorial treatment**

From: (Pollyea et al., 2014)

apoptotic signals than the bulk tumor (Hanahan & Weinberg, 2000; Signore, Ricci-Vitiani, & De Maria, 2013). Therefore, inhibiting these anti-apoptotic mechanisms is a method by which to directly potentiate the effect of chemotherapy.

Excitingly, in multiple instances LSCs have been shown to depend more highly on mechanisms of suppressing apoptosis than normal HSCs, potentially as a result of their increased rate of proliferation and accumulation of genetic aberrations (Guzman et al., 2002). One such example is nuclear factor kB (NF-kB), which has been shown to be constitutively active in LSCs but not normal HSCs (Guzman et al., 2001). NF-kB gene expression is also induced upon exposure to anthracyclines, presumably as a cell survival response (Laurent et al., 2001). The proteasome inhibitor MG-132 and NF-kB-specific inhibitors such as parthenolide bortezomib induce apoptosis in AML and CML LSCs while sparing normal HSCs (Guzman et al., 2002, 2005). Additionally, LSCs express lower levels of FAS and FAS ligand, which is an important pro-apoptotic death receptor, with decreased sensitivity to FAS-induced apoptosis (Costello et al., 2000). Low FAS expression can be overcome with synthetic FAS ligand mimetics such as Apo010, which increases survival of glioma tumor-bearing mice both as a single agent and in combination with other cytotoxic drugs (Eisele et al., 2011).

There are also examples of improving chemotherapeutic efficacy by directly targeting the pro-survival Bcl-2 family of proteins. Multiple Bcl-2 family members, including Bcl-2, Bcl-xL, and Mcl-1 are upregulated in LSCs and high Bcl-2 expression is associated with poor response to therapy in AML (Campos et al., 1993; Mak et al., 2012). Loss-of-function studies of Bcl-2 also demonstrates its expendability for HSC maintenance and self-renewal (Matsuzaki et al., 1997). Therefore, the therapeutic potential of inhibiting Bcl-2 has been extensively explored

(Signore et al., 2013). In one study, the Bcl-2 inhibitors ABT-263 and obatoclax were shown to preferentially induce LSC cell death with no significant impact on normal $CD34^+$ progenitors, potentially through dependence upon oxidative phosphorylation rather than glycolysis in LSCs (Lagadinou et al., 2013). All-*trans* retinoic acid (ATRA) has also been shown to increase AML cell chemosensitivity through down-regulation of Bcl-2, particularly in CD34-negative AML. (Bradbury, Aldington, Zhu, & Russell, 1996).

Microenvironmental changes in the BM may also render LSCs more resistant to apoptosis. LSCs have been shown to preferentially home to niches in the bone marrow microenvironment that protect them from apoptosis (Ishikawa et al., 2007). CD44, a ubiquitously expressed cell surface receptor that mediates cell-cell adhesion has been shown to be upregulated in AML and is associated with poor prognosis (Bendall, Bradstock, & Gottlieb, 2000; Legras et al., 1998). Antibody therapies directed at CD44 demonstrate specificity in inhibiting LSC trafficking to supportive microenvironments (Jin, Hope, Zhai, Smadja-Joffe, & Dick, 2006). Therefore, anti-apoptotic stimuli can be both cell-intrinsic and cell-extrinsic to the LSC, with multiple nodes that can potentially be leveraged for therapeutic intervention (**Figure 1-6**).

Inflammation

The inflammatory nature of the tumor microenvironment has also been postulated to promote leukemic progression and drug resistance (de Visser & Jonkers, 2009). Normal physiological inflammation, which is a combination of pro-inflammatory molecule synthesis and inflammatory cell mobilization and recruitment, is an important step for wound healing and tissue repair (Zlotnik & Yoshie, 2000).

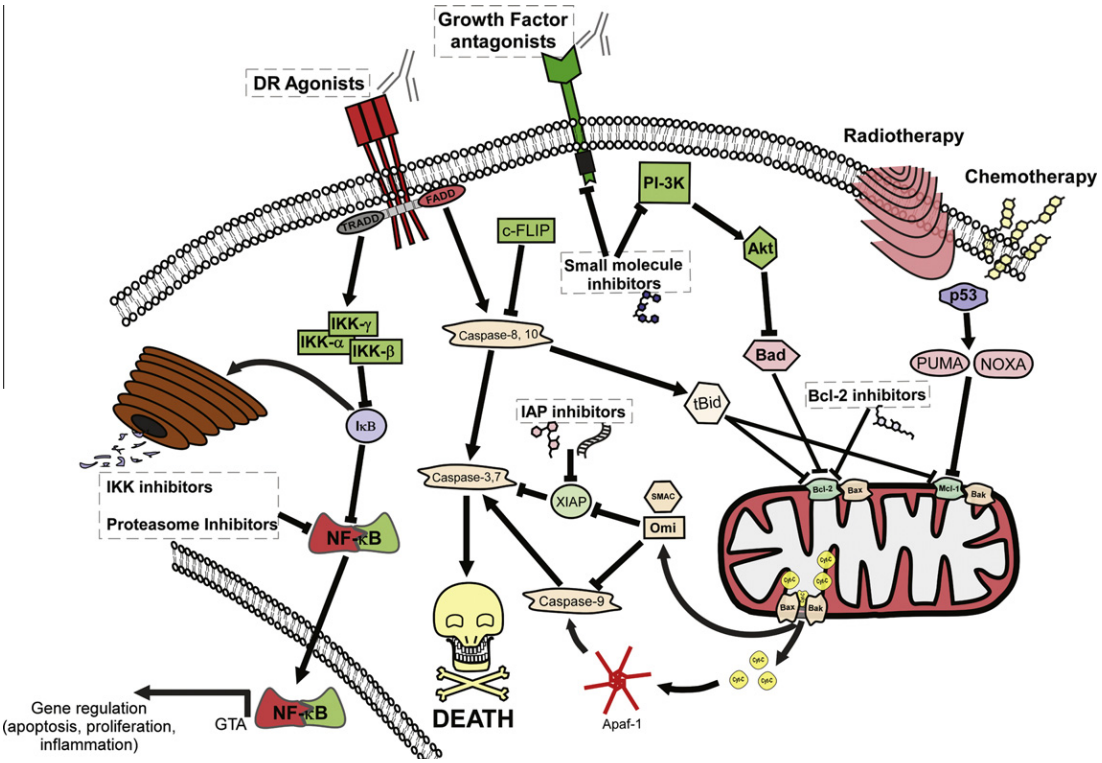


Figure 1-6: Inhibition of cell-intrinsic/extrinsic apoptotic pathways

From: (Signore et al., 2013)

Conversely, inflammation in the context of tumorigenesis is associated with aberrant expression of elevated and/or systemic levels of inflammatory cytokines such as interleukins and MCP-1 (Balkwill, Charles, & Mantovani, 2005). Doxorubicin treatment also induces MCP-1, TNFa, and IL-8 in lung carcinoma cell lines, highlighting a potential link between inflammatory mediators and response to chemotherapy (Niiya et al., 2003). Inflammation has been shown to influence several other chemoresistance pathways as well, including modulation of ABC-family transporters and CXCR4-dependent apoptosis (de Visser & Jonkers, 2009; Hartmann, Burger, Glodek, Fujii, & Burger, 2005). The extent of inflammation generally correlates with poor clinical prognosis in many cancer types (Ueno, Toi, & Saji, 2000). Together, these studies establish a cyclical link between inflammation, chemotherapy, and induction of chemoresistance.

While many reports interrogating the role of inflammatory processes in chemoresistance were conducted in solid tumor systems, some evidence has also been generated in the context of hematological malignancies. PKCζ and NF-kB, have been shown to elicit chemoprotection in follicular cell lymphoma and B-cell non-Hodgkin's lymphoma, respectively (Leseux et al., 2008; Vega, Jazirehi, Huerta-yepez, & Bonavida, 2005). PKCζ has a demonstrated role in the regulation of NF-kB translocation through IKK signaling complex activation (Bourbon, Yun, & Kester, 2000). NF-kB, which has a clear role in regulation of apoptotic induction in LSCs, also promotes cytokine production, including the positive LSC growth-regulator IL-6 (Brasier, 2010). NF-kB activation can also induce cyclin D gene transcription and cell cycle progression, c-myc activation, and remodeling of the extra-cellular matrix (Pham, Tamayo, Yoshimura, Lo, & Ford, 2003; Takebayashi et al., 2003). The multifaceted role of PKCζ and NF-kB may help explain the broad observations

concerning the effect of inflammation on chemoresistance (**Figure 1-7**).

Multiple studies have implicated inflammation in control of the tumor microenvironment and bulk tumor growth, yet little is currently known regarding the direct effect of inflammatory processes on LSCs. CML LSCs that localize to adipose tissues exhibit a pro-inflammatory phenotype, with strong upregulation of several inflammatory cytokines including IL-6, TNF-α, and CXCL1/2/3. These adipose-resident LSCs express high levels of CD36, are quiescent, and exhibit markedly higher resistance to chemotherapy (H. Ye et al., 2016).

Eicosanoids such as prostaglandins and leukotrienes, synthesized by cyclooxygenase (COX) and 5-lipoxyganase enzymes (5-LO), respectively, have demonstrated roles in inflammation and cancer (D. Wang & Dubois, 2010). Interestingly the COX inhibitor indomethacin was shown to induce a 100-fold decrease in AML initiating cells in secondary recipients, potentially through abrogation of Ctnnb1 activity (Y. Wang et al., 2010). Our studies have also identified 5-LO as being upregulated in AML patients upon exposure to induction therapy (see **Chapter Two**). We directly show that reduction in 5-LO leukotriene-synthetic capacity improves leukemic cell elimination *in vitro* and *in vivo* (see **Chapter Three**). Our data, together with studies performed in solid tumor stem cell maintenance and other myeloid neoplasms, identifies a role of canonical inflammatory mediators in the regulation of chemoresistance.

Epigenetic modification

Both DNA hyper- and hypo-methylated states have been linked to patient outcomes in AML. Multiple recurrently mutated or translocated genes involved in

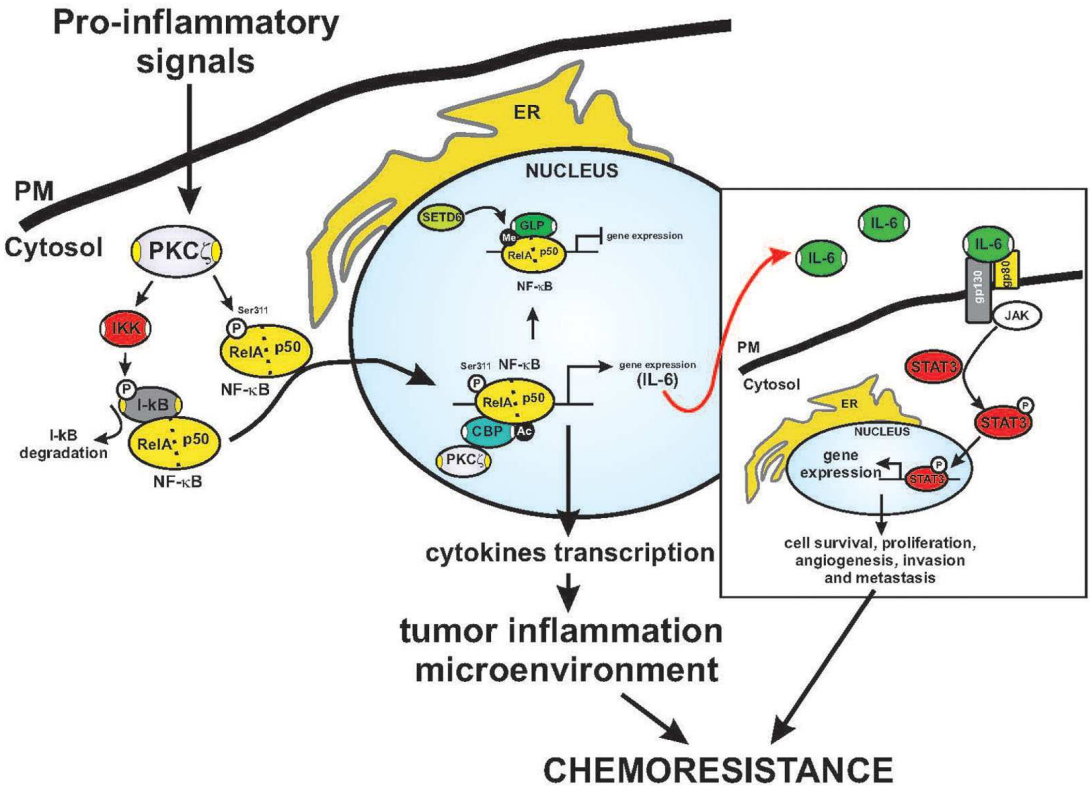


Figure 1-7: Pro-inflammatory signals in chemoresistance

From: (Rimessi, Patergnani, Ioannidi, & Pinton, 2013)

epigenetic regulation have been identified in AML, including: the DNA methylating protein DNMT3A, demethylating protein TET2, α-ketoglutarate converter IDH1/2, and histone modifiers HDAC2/3, EZH2, MLL1, and CREBBP (Wouters & Delwel, 2016). Mutations in these proteins leads to broad changes in global transcriptional programs, which, when combined with other oncogenic drivers such as Flt3-ITD or Nras mutations, is sufficient for the onset of AML (Shih et al., 2015). Commonly observed mutated epigenetic modifiers in AML are described here.

Mutations in TET2 are observed in 8% to 27% of AML patients, which typically leads to poorer prognosis, particularly in patients with intermediate-risk AML (Metzeler et al., 2011). Loss-of-function TET2 mutations result in global hypermethylation that overlaps between patients, suggesting that this pattern of aberrant methylation is not random (Busque et al., 2012). IDH1 and IDH2 mutations are also commonly observed in AML, with missense mutations in 5% to 16% and 6% to 19% for IDH1 and IDH2, respectively. Mutant IDH1/2 results in the synthesis of an aberrant metabolite, 2-hydroyglutarate, that has been shown to directly compete with α-ketoglutarate, resulting in TET inhibition (Ward et al., 2010). However, the clinical impact of IDH mutations are conflicting, with several large cohort studies demonstrating discrepant prognostic value (Abdel-wahab & Levine, 2013).

DNMT3A is mutated in 6% to 36% of AML patients (Voigt & Reinberg, 2013b). Mutation of DNMT3A appears to be an early event in leukemogenesis, as some patients have presented with T-lymphocytes harboring mutations in the gene, indicating presence of DNMT3A mutation in very early hematopoietic precursors that can give rise to both myeloid and lymphoid lineages (Shlush et al., 2014). Recent reports have showed that the DNMT3A R882H mutation, the most common DNMT3A mutation in patients, promotes chemoresistance through impaired DNA

damage sensing and chromatin remodeling capacities (Guryanova et al., 2016). AML patients with DNMT3A R882H mutation demonstrate poor response to standard-dose induction therapy, although better overall survival upon dose-escalation (Sehgal et al., 2015). DNMT3A transcript and protein were also shown to be upregulated in response to increasing doses of doxorubicin in human colorectal cell lines, with silencing of DNMT3A resulting in a larger percentage of senescent cells in response to doxorubicin treatment (Zhang et al., 2011). Together, these studies highlight DNMT3A as a chemotherapy-responsive gene that abrogates response to therapy *in vitro* and *in vivo*.

The reversible nature of epigenetic modifications makes the inhibition of histone modifiers or proteins that maintain DNA methylation an attractive strategy for therapeutic development. Indeed, several clinical trials investigating inhibitors of mutant DNMTs and IDH1/2 are currently on going (**Table 1-4**). Two DNA methyltransferase inhibitors, 5-azacytidine and decitabine, are currently approved for the treatment of MDS and low blast burden AML. These compounds reverse global hypermethylation, which results in improved patient outcomes, with CR rates of 18% and 24% for azacytidine and decitabine, respectively (Cashen, Schiller, O'Donnell, & DiPersio, 2010; Fenaux et al., 2010). Together these studies implicate aberrant epigenetic modification as a driver of leukemic transformation, however the role of methylation changes in LSC chemoresistance is just beginning to be investigated.

Drug development

Over the past decade, significant effort has been applied to the identification and inhibition of LSC-specific molecules (**Table 1-5**), many of which have been

Table 1-4: Clinical development of inhibitors for epigenetic modifiers

Class of epigenetic regulator	Target	Compound	Phase of development
DNA methyltransferase	DNMTs	Azacitidine	Approved (see text)
		Decitabine	Approved (see text)
		Rationally designed novel inhibitors	Preclinical and clinical[94,95]
Regulator of methylation	IDH1, IDH2	Inhibitors of mutant IDH1/2	Clinical trials ongoing with compounds including IDH305 (ClinicalTrials.gov identifier: NCT02381886; targeted at IDH1 R132 mutation), AG-221 (NCT01915498; targeted at mutant IDH2), AG-120 (NCT02074839; targeted at mutant IDH1)
Histone lysine acetyltransferase	CREBBP (CBP)	CREBBP inhibitor	Preclinical[96]
	EP300 (p300)	EP300 inhibitor	Preclinical[97]
Histone deacetylase	HDACs	HDAC inhibitors	Several clinical trials ongoing, often in combination with other treatment modalities (eg, with DNMT inhibitors [examples ClinicalTrials.gov identifiers NCT01617226 and NCT00867672], conventional chemotherapy [example NCT01802333], or in conjunction with allogeneic stem cell transplantation [examples NCT01451268 and http://www.hovon.nl/studies/studies-per-ziektebeeld/aml.html?action=showstudie&studie_id=104&categorie_id=4])
Histone acetyl reader	Bromodomain containing proteins (BET proteins)	BET inhibitors	Several clinical trials ongoing with compounds including OTX-015 (ClinicalTrials.gov identifier: NCT01713582), CPI-0610 (NCT02158858), TEN-010 (NCT02308761), GSK525762 (NCT01943851)
Histone lysine methyltransferase	EZH2	EZH2 inhibitors	Preclinical[98,99]
	MLL-complexes	DOT1L inhibitors	Clinical trial with compounds including EPZ-5676 (ClinicalTrials.gov identifier: NCT01684150)
		Inhibitors of MLL-Menin interface	Preclinical[100]
		Inhibitors of MLL-LEDGF interface	Preclinical[101]
Histone lysine demethylase	LSD1	LSD1 inhibitors	Clinical trials with compounds including GSK2879552 (ClinicalTrials.gov identifier: NCT02177812) and tranylcypromine in combination with tretinoine (NCT02261779)
	Jumonji family of KDMs	Small molecular inhibitors competitive for 2-oxoglutarate	Preclinical[102,103]
Histone arginine methyltransferase	PRMTs	PRMT inhibitors	Preclinical[104,105]

From: (Wouters & Delwel, 2016)

Table 1-5: LSC-specific markers for therapeutic development

	Antigen	Function/Characteristics	Expression		Reference
			LSCs	*HSCs*	
Cell surface markers	CD123	High affinity IL-3 receptor (IL-3α)	+	-	Jordan et al. (2000)
	CD47	Ligand for SIRPα, inhibits phagocytosis	+	-	Majeti et al. (2009)
	CD96	Activation of Antibody dependent cell-mediated toxicity	++*	+	Hosen et al. (2007)
	CD32	Fc-g receptor 2 (FCGR2)	+	-	Saito et al. (2010)
	CD25	High-affinity IL-2 receptor (IL2Rα)	+	-	Saito et al. (2010)
	CD44	Facilitates adhesive interactions, key regulator of AML-LSCs homing to microenvironmental niches	++*	+	Jin et al. (2006)
	CXCR4	Cell membrane receptor, contributes to SDF-1α/ CXCR4 interactions	++*	+	Spoo et al. (2007)
Intracellular markers	AurA	Mitotic serine/threonine kinases that play a role in cytokinesis during mitosis and cell division	++*	+	Ochi et al. (2009)
	Mcl-1	Plays a critical role in maintenance and survival of LSCs	++*	+	Yoshimoto et al. (2009)
	TIM-3	Regulator of macrophage activation , role with complement-dependent and antibody dependent cell-mediated cellular cytotoxic activities	+	-	Kikushige et al. (2010)
	NF-κB	Transcription factor, responsible for LSC antiapoptotic activity	+	-	Guzman et al. (2001)

From: (Al-Mawali, 2013)

discussed above. While LSC-targeted therapies for AML are only recently being developed, there are several analogous examples of LSC targeting in other myeloid neoplasms that may help guide the drug development process. Stem cells in CML have been shown to be relatively insensitive to imatinib (Graham et al., 2002). Administration of interferon-α (IFN) demonstrated a slower, but more durable response than imatinib, suggesting superior long-term efficacy of drugs that target LSCs (Angstreich et al., 2005). In multiple myeloma, drugs such as bortezomib lenalidomide do not target myeloma stem cells and do not confer long-term cure. Alternatively, rituximab, an anti-CD20 antibody, has shown specificity to myeloma stem cells but is ineffective as a single agent (Zojer, Kirchbacher, Vesely, Hübl, & Ludwig, 2006). This underscores the promise of combining therapies that debulk the tumor with LSC-targeted therapies for elimination of the complete leukemic cell population.

Finally, although LSCs are likely the most chemoresistant cell population in AML, there are still distinct advantages of rendering the bulk leukemia more sensitive to chemotherapy as well. One can imagine a case where a general chemoresistance mechanism is therapeutically targeted in combination with induction chemotherapy. Two examples help illustrate the benefit of such a treatment regimen: 1) A young adult with intermediate-risk AML is treated with standard concentrations of induction therapy in combination with the chemosensitizing agent. A greater reduction in blast burden is observed than induction therapy alone, resulting in undetectable MRD and prolonged remission. 2) Reduced concentrations of induction therapy are used in combination with the chemosensitizing agent to treat an elderly AML patient, reaching the same level of efficacy with better tolerance and more effective hematopoietic recovery, resulting in improved overall survival. Both cases would be attractive

clinically, highlighting the potential benefits of leukemic blast chemosensitization.

In sum, it is likely that multiple processes act simultaneously to render LSCs resistant to chemotherapy. Understanding the overlap between these processes is therefore required. The timing of conventional induction therapy with drugs directed at eliminating LSCs will likely be important for optimal efficacy, which are expected to relate to the mechanism of action of said therapy. While genetic mutations likely increase the baseline resistance of leukemic cells to therapy, it is evident that temporal changes in gene expression following exposure to therapy may also be telling of cellular mechanisms of chemoresistance. Therefore, a better understanding of how LSCs react to chemotherapy at the transcriptional level is required.

ARACHIDONATE 5-LIPOXYGENASE

To this end, we have initiated studies that have identified multiple upregulated genes in AML patient leukemic cells, immediately following induction therapy. Among these genes, arachidonate 5-lipoxygenase (5-LO) was one of the most upregulated genes, with maximal expression observed in LSCs (see **Chapter Two**). Given the link between inflammatory processes and protection of LSCs from chemotherapy, combined with promising pre-clinical 5-LO inhibitor studies in the context of CML (Y. Chen, Hu, Zhang, Peng, & Li, 2009; de Visser & Jonkers, 2009), we were interested in investigating the role of 5-LO in AML chemoresistance.

Role in inflammation

Arachidonic acid is an abundant biologically active lipid derivative that is

enzymatically processed into the eicosanoids, leukotrienes and prostaglandins, by 5-LO and cyclooxygenase (COX), respectively (**Figure 1-8**). Both 5-LO and COX have demonstrated roles in inflammation and carcinogenesis in several tumor models (de Groot, de Vries, Groen, & de Jong, 2007; Dubois et al., 1998; Eyberger, Dondapati, & Porter, 2006), with inhibition of COX potentially reducing the frequency of AML initiating cells (Y. Wang et al., 2010). However, for the sake of simplicity only 5-LO and leukotrienes will be elaborated upon here.

5-LO is the primary catalytic enzyme involved in the synthesis of leukotrienes. Through interaction with the 5-LO activating protein (FLAP), 5-LO converts arachidonic acid into 5-HETE. 5-LO then mediates a second catalytic reaction to convert 5-HETE into the unstable leukotriene intermediate, LTA_4. Biologically active LTB_4 is further converted from LTA_4 by LTA_4 hydrolase or to the cysteinyl leukotriene (cysLT) LTC_4 by LTC_4 synthase. LTC_4 is then further processed into LTD_4, which is sequentially metabolized to LTE_4 (Samuelsson, 1983).
LTB_4, as well as the cysLTs LTC_4, LTD_4, and LTE_4 are all biologically active signaling molecules. They elicit their effect by binding to specific receptors in either an autocrine or paracrine fashion. The leukotriene receptors BLT1 and BLT2 (for LTB_4), and CysLT1 and CysLT2 (for LTC_4, LTD_4, and LTE_4), are g-protein coupled receptors (GPCRs) expressed on a wide variety of cell types, with the exception of BLT1 and CysLT1, which are expressed exclusively on leukocytes.

Interestingly, leukotrienes can be produced by a single cell or through a process called transcellular biosynthesis, in which LTA_4 is released by neutrophils at inflammatory sites and converted into LTB_4, LTC_4, and LTD_4 by epithelial and endothelial cells (Folco & Murphy, 2006). Leukocytes can also generate leukotrienes from arachidonic acid released from epithelial cells

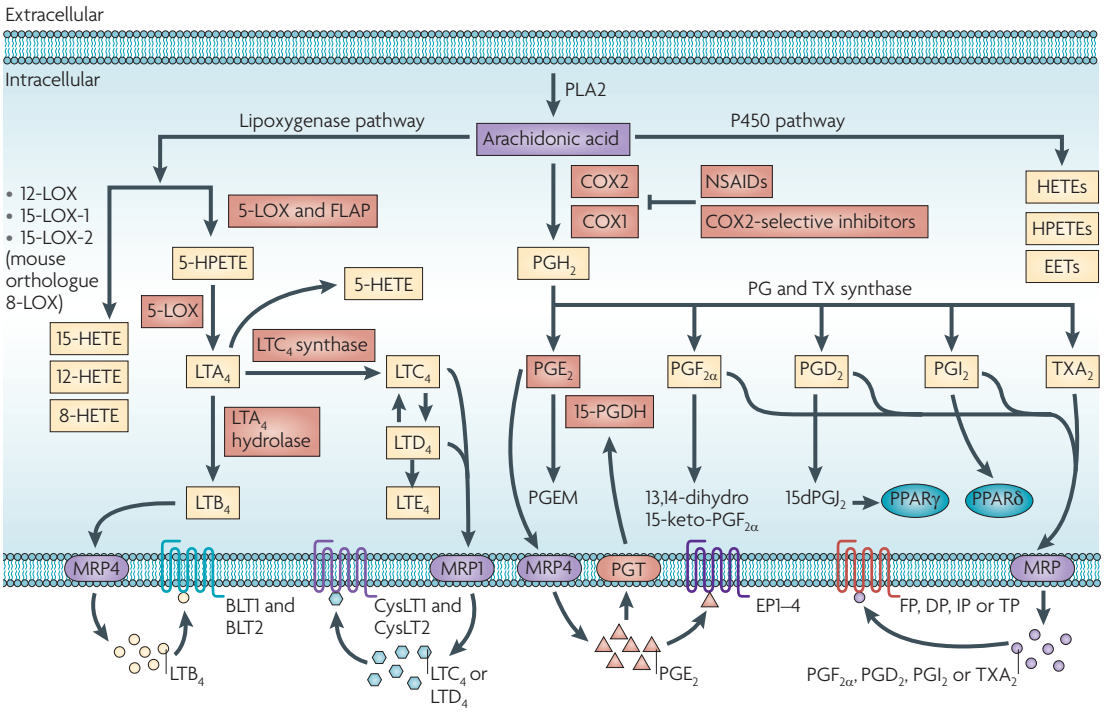


Figure 1-8: 5-LO and leukotriene synthesis

From: (D. Wang & Dubois, 2010)

(Zarini, Gijón, Ransome, Murphy, & Sala, 2009). Through this process immune cells generate an excess of leukotrienes, which in turn amplifies the inflammatory response. How this process occurs in the inflammatory tumor microenvironment, particularly in the context of chemotherapy, is currently unknown.

The role of 5-LO, particularly in respect to inflammation and immune response, is well characterized in the hematopoietic system. Leukotrienes are potent broncho- and vasoconstrictors and are important for immediate hypersensitivity reactions such as asthma (Ogawa & Calhoun, 2006; Piper et al., 1991). Leukotrienes also mediate adhesion of neutrophils to endothelial cells, extravasation of immune cells, and are strong chemotactic agents for eosinophils and monocytes (Corey, 1982). LTB_4 and cysLTs mediate slightly different biologic response, with cysLTs being major mediators of airway anaphylaxis and LTB_4 influencing greater effect on cell mobilization and recruitment.

Endogenous and exogenous leukotrienes also mediate macrophage elimination of foreign microbes through enhanced effector function and secretion of pro-inflammatory molecules (Medeiros et al., 2004; Peres et al., 2007). Leukotrienes stimulate leukocyte migration and activation, $CD4^+$ T cell chemotaxis, and primary and secondary immune responses against a variety of pathogens (Medeiros, Silva, Malheiro, Maffei, & Faccioli, 1999; Tager et al., 2003). Studies in 5-LO knockout mice demonstrate an impairment in both innate and adaptive immune responses during fungal infection (Secatto et al., 2012). It is therefore clear that leukotrienes potentiate many biological processes relating to the function and recruitment of blood cells.

Effects on hematopoiesis

5-LO and its products have also been shown to directly affect myelopoiesis. Exogenous administration of LTB_4 to mononuclear BM cells in culture leads to an increase in granulocyte-macrophage colony formation (Claesson, Dahlberg, & Gahrton, 1985). Inhibition of 5-LO also inhibited colony-stimulating factor (CSF)-induced colony formation in both murine and human BM cells (Ziboh, Wong, Wu, & Yunis, 1986). CysLT binding to their cognate receptors on human monocyte and smooth muscle cells activates distinct gene expression changes, with elevated expression of FOSB, EGR2, EGR3, NR4A2, and TSC22D3 transcription factors (Eaton, Nagy, Pacault, Fauconnier, & Bäck, 2012; Lundeen, Sun, Karlsson, & Fourie, 2006; Woszczek et al., 2008). Many of these induced genes have themselves been linked to various inflammatory processes (Kharbanda et al., 1991; Thompson et al., 2006). Furthermore, exogenous TGF-β and/or GM-CSF were shown to upregulate 5-LO expression in HL-60 cells (M. Brungs, Radmark, Samuelsson, & Steinhilber, 1994). Differentiation of U937, HL-60, and Monomac-6 cells with DMSO also upregulated 5-LO expression (Bennett, Chiang, Monia, & Crooke, 1993; Martina Brungs, Radmarkt, Samuelssont, & Steinhilber, 1995).

Leukotrienes have been shown to elicit effects on more primitive cells of the blood as well. LTB_4 promotes the proliferation, cell survival, and differentiation of umbilical cord blood (CB) $CD34^+$ HSPCs cultured *ex vivo*. Conversely, LTB_4 receptor blockade with CD105696 increased the self-renewal of $CD34^+$ HSPCs in culture (Chung, Kim, Mun, & Ahn, 2005). LTD_4 also promoted primary HSC proliferation, potentially through phosphorylation of ERK/MAPK. Interestingly, a 90% reduction in cysLT synthesis was observed when bone marrow stromal cells were

co-cultured with $CD34^+$ HSPCs, suggesting a negative regulatory mechanism between HSPCs and the microenvironment (Boehmler et al., 2009). LTB_4 and LTD_4 have been shown to activate overlapping gene expression programs and similar promotion of chemotaxis in monocytes, indicating redundant but cooperative biological functions (L. Y. Chen et al., 2011). Together, these studies demonstrate that leukotrienes can affect multiple processes in hematopoietic function and differentiation, at both the physiological and transcriptional levels.

5-LO inhibition and leukemia

The role of leukotrienes in inflammation, particularly in inflammatory disorders, has led to the development of leukotriene inhibitors since the early 90's. Several examples of FDA-approved inhibitors of leukotrienes include the leukotriene receptor inhibitors monteleukast and zafirlukast, and the 5-LO inhibitor zileuton (Drazen, Silverman, & Lee, 2000). All three drugs reduce airway obstruction and granulocyte chemotaxis, and are approved for the treatment of mild-to-moderate asthma (Ogawa & Calhoun, 2006; Sladek et al., 1990). 5-LO has also been linked to atherosclerosis, another chronic inflammatory disorder, heightening interest in its inhibition (Mehrabian et al., 2002). Moreover, the involvement of leukotrienes in tumorigenesis has become more clear in recent years (D. Wang & Dubois, 2010). Studies in a variety of solid tumor types, including human lung, breast, and pancreatic cancers, have reported a leukotriene-mediated promotion of tumor cell growth, which is reversed upon both 5-LO and leukotriene receptor inhibition (Bishayee & Khuda-Bukhsh, 2013; Xiaoxin Chen et al., 2003; Wenger et al., 2002). This has expanded the potential applications of 5-LO inhibition; however, none have been realized clinically.

Given the broad range of influence 5-LO has over the hematopoietic system, it is logical that leukemic cells also co-opt 5-LO biology to their advantage. Indeed, the first hints of this came from 5-LO knockout studies in the context of CML. 5-LO gene expression was shown to be upregulated by BCR-ABL. BCR-ABL 5-LO$^{-/-}$ mice failed to develop CML, with 5-LO$^{-/-}$ LSCs exhibiting a strong disadvantage when competitively transplanted in a 1:1 ratio with 5-LO$^{+/+}$ LSC (Y. Chen, Li, & Li, 2009). BCR-ABL leukemic mice treated with zileuton have improved duration of survival compared to placebo or imatinib treated mice (Y. Chen, Hu, et al., 2009). Gene expression profiling of Lin^{-}c-Kit^{+}Sca-1^{+} cells from 5-LO$^{-/-}$ leukemic mice demonstrated upregulation of Msr1, leading to potential impairments in LSC function through altered PI3K-AKT and β-catenin signaling (Y. Chen et al., 2011). A high level of LTB_4 induction following imatinib therapy is also associated with poor response rates in patients with CML (Lucas, Harris, Giannoudis, McDonald, & Clark, 2014). These studies implicate 5-LO in the self-renewal of chronic phase CML stem cells, however direct interrogation of the mechanisms by which this occurs are incomplete.

In AML, the RUNX1-ETO9a gene fusion was shown to upregulate 5-LO expression, with loss of 5-LO reducing the activity of RUNX1-ETO9a, MLL-AF9, and PML-RARα leukemic cells *in vitro* (DeKelver et al., 2013). AML LSCs exhibit high expression of receptors for both LTB_4 and cysteinyl leukotrienes, as well as elevated 5-LO expression compared to normal hematopoietic stem and progenitor cells (Gentles et al., 2012; Seita et al., 2012; D. Wang & Dubois, 2010). Studies in the context of a PML/RARα-positive model of AML demonstrated that pharmacologic inhibition of 5-LO with CJ-13,610 reversed PML/RARα activation of Wnt signaling, suggesting a link between 5-LO catalytic inactivation and inhibition of Wnt as a

leukotriene-independent mechanism of LSC survival (Roos et al., 2014). However, these studies did not account for potential off-target effects of small molecule addition, nor did they assess the impact of 5-LO depletion on LSC chemoresponses *in vivo*. Therefore, additional studies that investigate the mechanism of 5-LO-medaited LSC chemoprotection are required.

OTHER PUTATIVE CHEMORESISTANCE GENES

Additional putative genes affecting chemoresistance elucidated from our studies include: The inflammatory molecules S100A8 and S100A9, the anti-microbial molecule lysozyme (LYZ), the cathepsin inhibitor cystatin F (CST7), and the fatty acid transporter CD36. These molecules all have described roles in the hematopoietic system, with their influence in AML beginning to be delineated. As we show that these proteins are upregulated in response to chemotherapy in AML patients, with knockdown sensitizing human AML cell lines to both Ara-C and DNR (see **Chapter Two**), a brief overview of their role in hematopoiesis and leukemia is given here.

Normal biological function

Serum levels of S100A8/9 are important biomarkers for a range of inflammatory processes, including acute lung inflammation, inflammatory bowel disease, and rheumatoid arthritis (Foell & Roth, 2004). S100A8/9 are expressed primarily by granulocytes, monocytes, and early macrophages (Vogl, Gharibyan, & Morozova-Roche, 2012). S100A8/9 have been shown to induce neutrophil chemotaxis and adhesion (Ryckman, Vandal, Rouleau, Talbot, & Tessier, 2003).

Together, they constitute 40% of total cytosolic protein in neutrophils, potentially highlighting a role in rapid Ca^{2+} neutralization in this cell type, however this is yet to be experimentally validated (Hessian, Edgeworth, & Hogg, 1993).

CST7 is a member of the family of cysteine protease (cathepsin) inhibitors, which are primarily responsible for mediating protein degradation in endolysosomal compartments (Mohamed & Sloane, 2006). CST7 is expressed predominantly on cells of the hematopoietic system, including $CD8^+$ T cells, natural killer (NK) cells, monocytes, and dendritic cells (Ni et al., 1998). It is thought that CST7 mediates immune response through inhibition of cathepsins L (immune cell adhesion) and C (granzyme activation) (Hamilton, Colbert, Schuettelkopf, & Watts, 2008; Magister et al., 2012).

Unlike S100A8/9 and CST7, CD36 expression is not restricted to hematopoietic cells, being present on not only phagocytes and erythroid precursors, but also hepatocytes, cardiomyocytes, and specialized epithelia of the breast, kidney, and gut (M Febbraio, Hajjar, & Silverstein, 2001). In monocytes and macrophages CD36 primarily functions as a bioactive lipid transporter, with CD36-null mice exhibiting abnormal plasma lipid profiles and resting hypoglycemia (Maria Febbraio et al., 1999; Han, Hajjar, Febbraio, & Nicholson, 1997). CD36-fatty acid interactions may contribute to the pathogenesis of metabolic disorders such as obesity and insulin resistance (Liang et al., 2004; Miyaoka et al., 2001)

LYZ is a secreted bacteriolytic enzyme in many tissues and body fluids with roles in the innate immune system. LYZ is highly expressed in hematopoietic cells, being found in granulocytes, monocytes, and macrophages, as well as their precursors in the BM (Dumoulin, Johnson, Bellotti, & Dobson, 1977). Furthermore, Lyz expression was observed in a subset of HSCs. Mice containing yellow fluorescence

protein knocked into the Lyz locus demonstrate complete hematopoietic reconstitution of $EYFP^+$ HSCs into irradiated recipients (M. Ye et al., 2003). This suggests that Lyz expressing HSCs can give rise to all hematopoietic lineages, however it is unclear how this HSC subset differs from the rest of the stem/progenitor pool.

Implications in tumorigenesis

Higher levels of S100A8 were found in mononuclear BM cells derived from patients with both primary and relapsed leukemia compared to healthy individuals or AML patients that achieved CR (Yang et al., 2012). Elevated expression of S100A8/9 in MLL-rearranged infant acute lymphoblastic leukemia (ALL) results in glucocorticoid resistance, a common treatment for this disease (Spijkers-Hagelstein et al., 2012). Conversely, knockdown of S100A8 enhances AML cell killing upon treatment with arsenic trioxide (Yang et al., 2012). S100A9 interaction with CD33, a cell surface receptor shown to be highly expressed on leukemic blasts, was demonstrated to drive myeloid-derived suppressor cell expansion and development of myelodysplastic syndrome (MDS) (Xianghong Chen et al., 2013).

High CST7 expression was shown to be a better than both ERK and p53 as a marker for tumor progression in colorectal cancer (Georgieva et al., 2008). Elevated CST7 expression was also associated with higher rates of liver metastasis and worse overall patient survival (Utsunomiya et al., 2002). Aberrant Runx2 expression in prostate cancer also induced CST7 gene activity (Baniwal et al., 2010). However, other studies have indicated that inhibition of cathepsins may be a method of inducing apoptosis in human leukemias (Stoka, Turk, Schendel, & Kim, 2001; Zhu & Uckun, 2000). CD36 expression was shown to mark a population of chemoresistant LSCs that

preferentially reside in gonadal adipose tissue (H. Ye et al., 2016). The extracellular matrix protein, thrombospondin-1 (TSP-1), an endogenous CD36 antagonist, was shown to prevent inflammatory lymphoangiogenesis through CD36 blockade in monocytic cells (Cursiefen et al., 2011).

Elevated levels of LYZ in plasma and urine is associated with a range of pathological disorders, and is considered a biomarker for monocytic leukemia. Indeed, in patients with myeloproliferative disorders, LYZ expression is elevated by a factor up to 4 (Osserman & Lawlor, 1966). By French-American-British (FAB) classification, diagnosis of acute myelomonoblastic leukemia is based on presence of 20% BM monocytes or serum lysozyme that exceeds the reference sample by three times. Indeed, measurement of serum LYZ alone could identify an AML patient with eosinophilia and no immunophenotypic or cytochemical features of monocytic differentiation (Moscinski, Kasnic, & Saker, 1991). However, the impact of LYZ expression on leukemogenesis, particularly in the context of LSC function, is unclear.

Together, the putative chemoresistance mediators identified by our studies have canonical roles in a variety of diverse biological processes. The majority of these genes are highly expressed in the hematopoietic system and underlie a number of diseases, including cancer. We highlight another role for these genes in LSC chemoresistance and suggest co-option of their inhibitors for improving chemoresponses in AML.

In sum, AML is characterized by genetic abnormalities that prevent normal myelopoiesis from running to completion. This partial differentiation pathway is driven by a population of quiescent, relatively chemoresistance LSCs. Multiple mechanisms of LSC chemotherapy evasion have been proposed, however our understanding of chemoresistance in AML is far from complete. In order for novel

LSC-targeted therapies to be generated, we must first understand how patient LSCs respond to chemotherapy *in vivo*. **Chapter Two** will focus on our interrogation of gene expression changes in LSCs, LPCs, and leukemic blasts sorted from patients undergoing induction therapy. **Chapter Three** will then delve into the characterization of one novel AML chemoresistance gene, 5-LO, including its role in mediating chemoresistance *in vitro* and *in vivo,* and the effect of 5-LO depletion on normal hematopoiesis.

CHAPTER TWO:
IDENTIFICATION OF PUTATIVE CHEMORESISTANCE GENES IN AML

INTRODUCTION

Acute myeloid leukemia (AML) arises as a result of immature myeloid cell expansion in the bone marrow resulting in replacement of normal hematopoietic stem/progenitor cells, eventually leading to ineffective hematopoiesis and serious complications from peripheral cytopenias. Despite the recent development of drugs targeting genetic lesions enriched in leukemia, including FLT3, CD44, and IDH1/2, treatment options for the majority of AML patients remain limited and outcomes are very poor (Estey, Levine, & Bob, 2015; Jin et al., 2006; Stone et al., 2005; Yu et al., 2003). Standard induction therapy – comprised of a combination of the nucleoside analog cytarabine (Ara-C) and an anthracycline such as daunorubicin (DNR) – induces complete remission (CR) in the vast majority of patients following induction therapy and has remained the clinical mainstay in AML therapy for nearly 30 years (Quigley et al., 1980; Roboz, 2011). However, primary resistance occurs in 10 to 40% of patients, with durable long-term cures achieved in only a minority of adult AMLs

(Rowe & Tallman, 2010; Thol, Schlenk, Heuser, & Ganser, 2015). Given the robustness of induction therapy, finding novel approaches to bolster the efficacy of this first line treatment has the potential to significantly improve patient outcomes.

AML is thought to arise from, and be maintained by, leukemic stem cells (LSCs) (Bonnet & Dick, 1997). Because LSCs are relatively therapy-resistant and can re-initiate disease after therapy, therapeutic targeting of LSCs represents an important hurdle in the treatment of leukemia. While several genes mediating chemoresistance in AML have been identified, mechanisms of resistance are far from complete (Eppert et al., 2011; Guryanova et al., n.d.; Krivtsov et al., 2006). Moreover, genes specifically mediating primary patient LSCs chemoresponses to induction therapy have not been directly evaluated. Classical methods used to identify potential resistance mediators based on gene expression profiling at the time of diagnosis, while useful, only offer a static glimpse into baseline gene expression and cannot necessarily capture how leukemic cells respond to chemotherapy *in vivo*. Therefore, new approaches which assess temporal gene expression changes in patient LSCs is an important unresolved question in AML biology.

In this chapter we characterize gene expression changes in paired diagnosis:post-induction LSC-enriched samples from AML patients in order to identify novel chemoresistance mediators. Our analysis has revealed LSC-specific gene expression changes in response to induction therapy. A portion of these genes are expressed at relatively low levels prior to therapy, with robust induction after chemotherapy. Indeed, many classical resistance genes including ATP-binding cassette transporters, BCL-2 related proteins, or regulators of self-renewal were not increased in response to chemotherapy in our study (Heidel et al., 2011; Tang et al., 2008), demonstrating the pitfalls of evaluating leukemic cells based only on

expression levels at diagnosis. Instead our data identify multiple previously unrecognized AML resistance genes including 5-LO, CD36, S100A8, S100A9, LYZ, and CST7, which we have validated as bona fide mediators of chemoresistance using *in vitro* and *in vivo* models. Together, these studies show the importance of directly evaluating LSC responses to chemotherapy and demonstrate the potential of combining targeted therapies with standard induction chemotherapy to improve clinical outcomes.

RESULTS

Patient LSCs exhibit unique chemo-responsive transcriptional profiles

To identify genes that exhibit altered expression following induction therapy, we sorted LSC-enriched (CD34+CD38-CD90-), leukemic progenitor cells (LPCs; CD34+CD38+), and CD34- cells from AML patient bone marrow aspirates, both before and 14 days following the initiation of standard "7+3" induction therapy. Patients in the study (N = 12) were heterogeneous with respect to age, cytogenetics, and response to therapy (**Table 2-1**). Total RNA was extracted from cell-sorted samples and transcriptome profiling was performed using Affymetrix U133 Plus 2.0 gene expression microarrays. Despite the sample heterogeneity, we identified distinct gene expression changes in all three cell populations (**Figure 2-1 A**). Treated and non-treated samples clustered by principle component analysis (PCA) (**Figure 2-1 B**). In total, we identified 5423 dysregulated genes in LSCs, LPCs, and CD34- cells following induction therapy ($P < 0.05$), 506 of which were unique to LSCs (**Figure 2-**

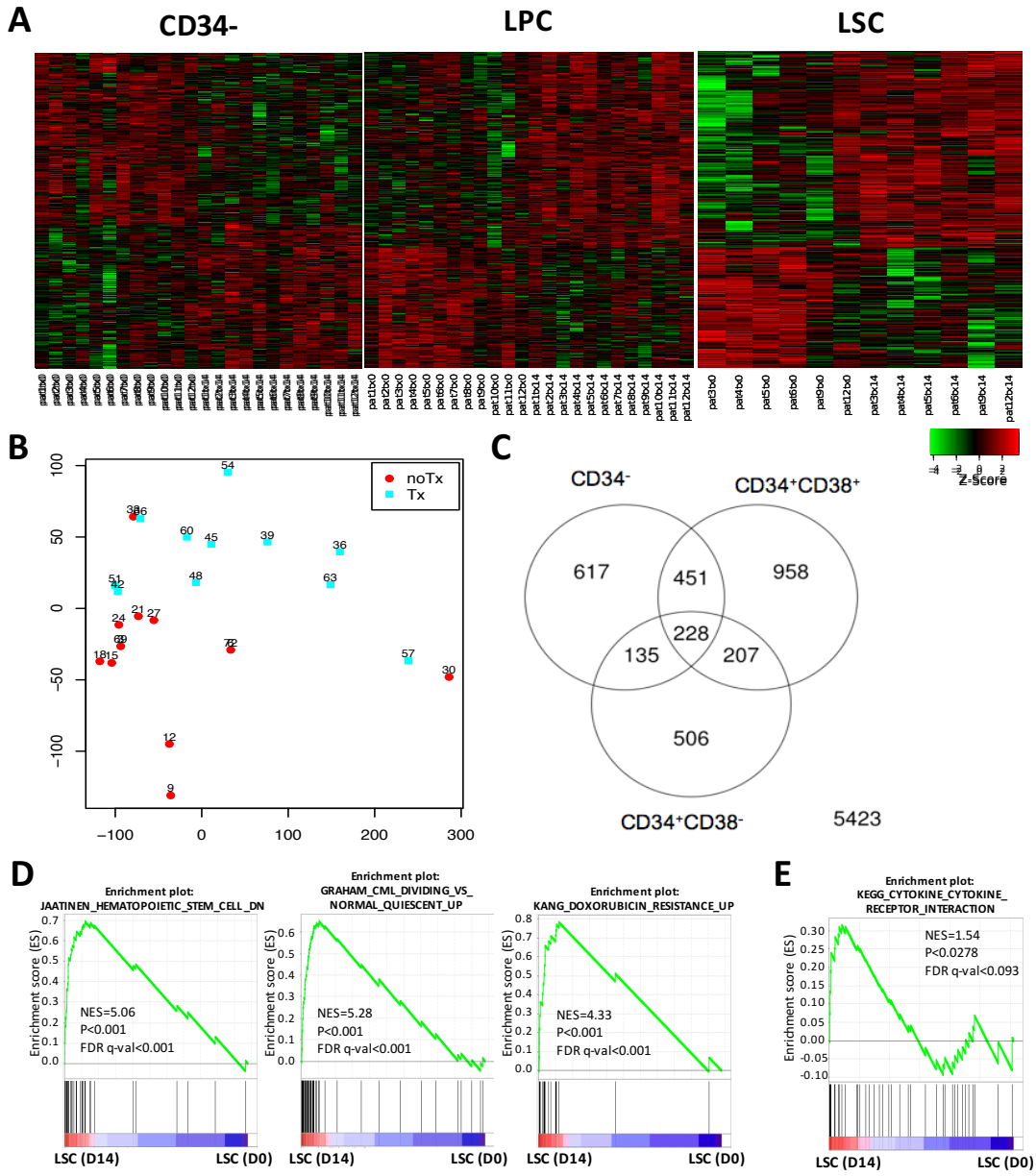
Table 2-1: **AML patient information**

#	Age	Sex	% blast (D0)	% blast (D14)
1	44	M	80%	51%
2	60	M	42%	28%
3	54	M	40%	86%
4	64	M	9%	14%
5	13	F	51%	9%
6	60	M	30%	17%
7	2	M	29%	NA
8	45	M	92%	94%
9	56	M	31%	25%
10	21	M	63%	15%
11	52	M	88%	79%
12	44	F	78%	40%

Patient information including blast counts assessed at the beginning (D0) and 14 days after the start (D14) of induction therapy.

<u>Figure 2-1:</u> LSC gene expression changes in patients undergoing induction therapy

(A) Heatmaps of top fold-change dysregulated genes ($P<0.05$) upon induction therapy in patient LSC (CD34+CD38-CD90-), LPC (CD34+CD38-), and blast (CD34-) cell populations. (B) Multi-dimensional scaling of patient LSC gene expression profiles before (D0, noTx, red circle) and after (D14, Tx, light blue circle). (C) Venn diagrams depicting overlap in differentially expressed genes following induction therapy in LSC, LPC, and blast cell populations. (D) GSEA and (E) KEGG pathway analysis of positive signature enrichment in differentially expressed patient LSC genes following induction therapy.


A
CD34-
LPC
LSC
B
noTx
Tx
C
CD34-
CD34+CD38+
CD34+CD38-
617
451
958
228
135
207
506
5423
Z-Score
D
Enrichment plot:
JAATINEN_HEMATOPOIETIC_STEM_CELL_DN
NES=5.06
P<0.001
FDR q-val<0.001
Enrichment plot:
GRAHAM_CML_DIVIDING_VS_NORMAL_QUIESCENT_UP
NES=5.28
P<0.001
FDR q-val<0.001
Enrichment plot:
KANG_DOXORUBICIN_RESISTANCE_UP
NES=4.33
P<0.001
FDR q-val<0.001
Enrichment score (ES)
LSC (D14)
LSC (D0)
E
Enrichment plot:
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION
NES=1.54
P<0.0278
FDR q-val<0.093

1 C). Gene set enrichment analysis (GSEA) of dysregulated genes in LSCs revealed enrichment of doxorubicin resistance, CML dividing, and downregulated HSC gene signatures (**Figure 2-1 D**). KEGG analysis demonstrated enrichment for cytokine-cytokine receptor signaling, adipocytokine signaling, and JAK/STAT signaling in LSCs (**Figure 2-1 E, Figure 2-2**). Interestingly, many of these pathways were enriched in LPC and CD34- cells as well, suggesting commonality in leukemic cell response to chemotherapy along the differentiation continuum (**Figure 2-2**). These analyses indicate that, while leukemic cells share commonalities in response to chemotherapy, there are discrete differences at the level of individual gene dysregulation.

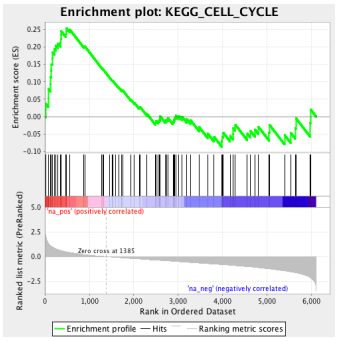
LSCs exhibit chemotherapeutic response involving multiple pathways

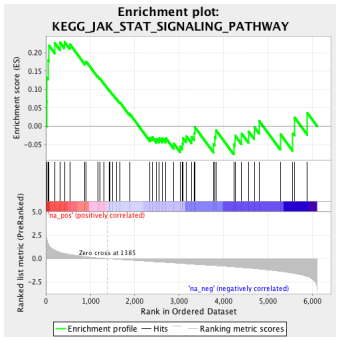
Comparison of our dysregulated genes from LSC, LPC, and CD34- populations revealed minimal enrichment in a curated gene set of known stemness regulators (**Figure 2-3**). As expected, the stemness regulators that are represented in our gene set exhibit distinct expression profiles for the cell types tested (**Figure 2-4 A**), which may be a general reflection of their differentiation potential (Stiehl et al., 2015). Interestingly, for all three cell populations, >85% of both known positive and negative stemness regulators do not significantly change ($P < 0.05$; **Figure 2-4 B**). Combined with our GSEA of LSC gene expression, this suggests that LSCs maintain their stem cell capacity while remaining primed for expansion immediately following induction therapy, which is distinct from published HSC gene signatures.

Figure 2-2: GSEA and KEGG pathways enrichment in sorted patient AML cell populations

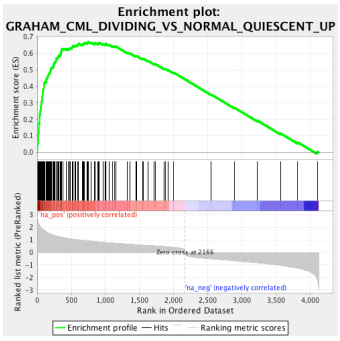
Additional GSEA and KEGG pathway enrichment plots for LSC (CD34+CD38-CD90-), LPC (CD34+CD38+), and blast (CD34+) blast cell populations. Signatures with positive enrichment are shown.

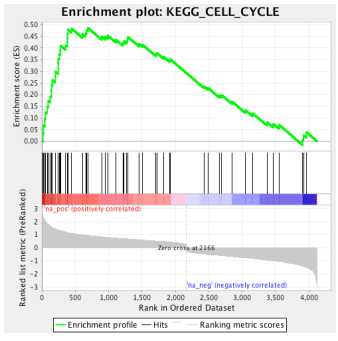
CD34+CD38-

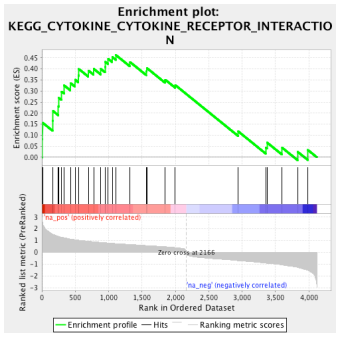


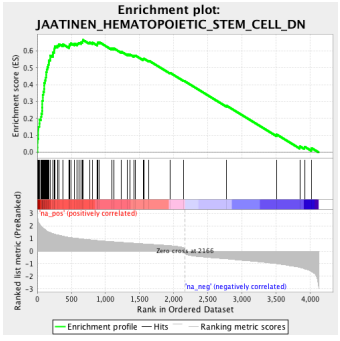


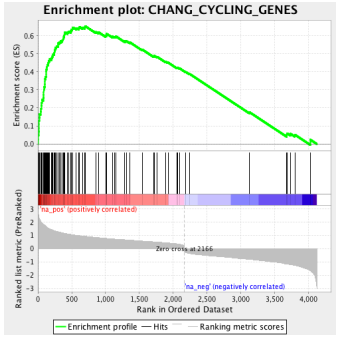
CD34+CD38+

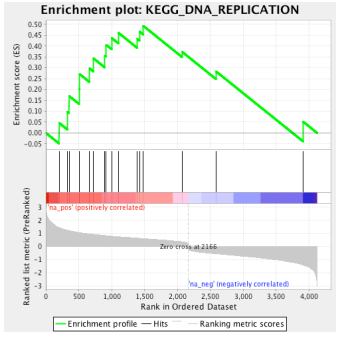




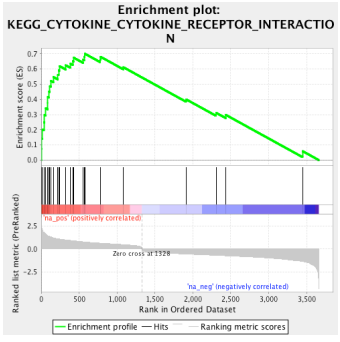


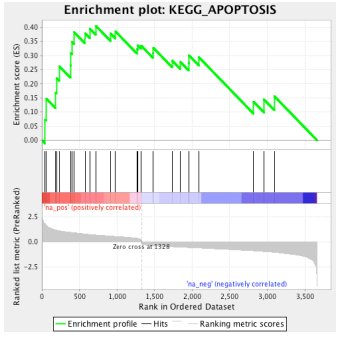


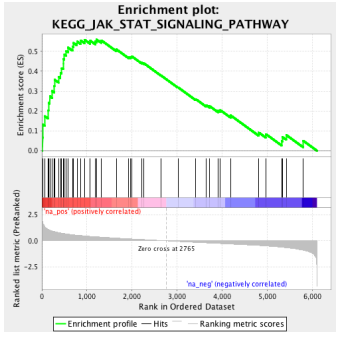


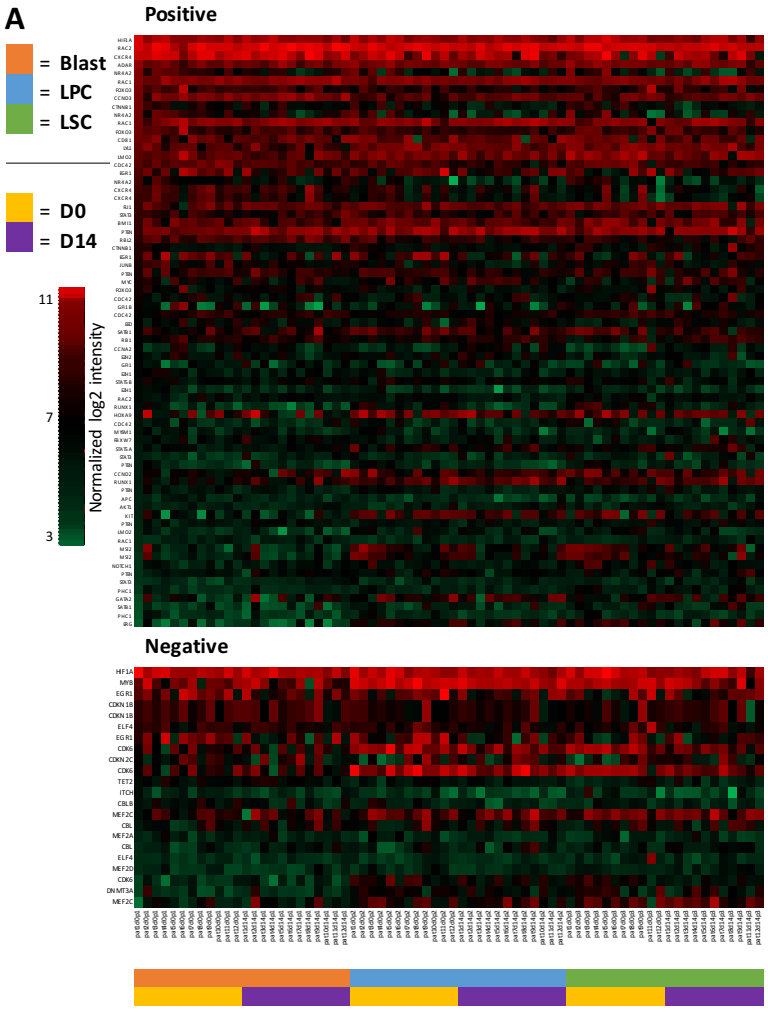


CD34-





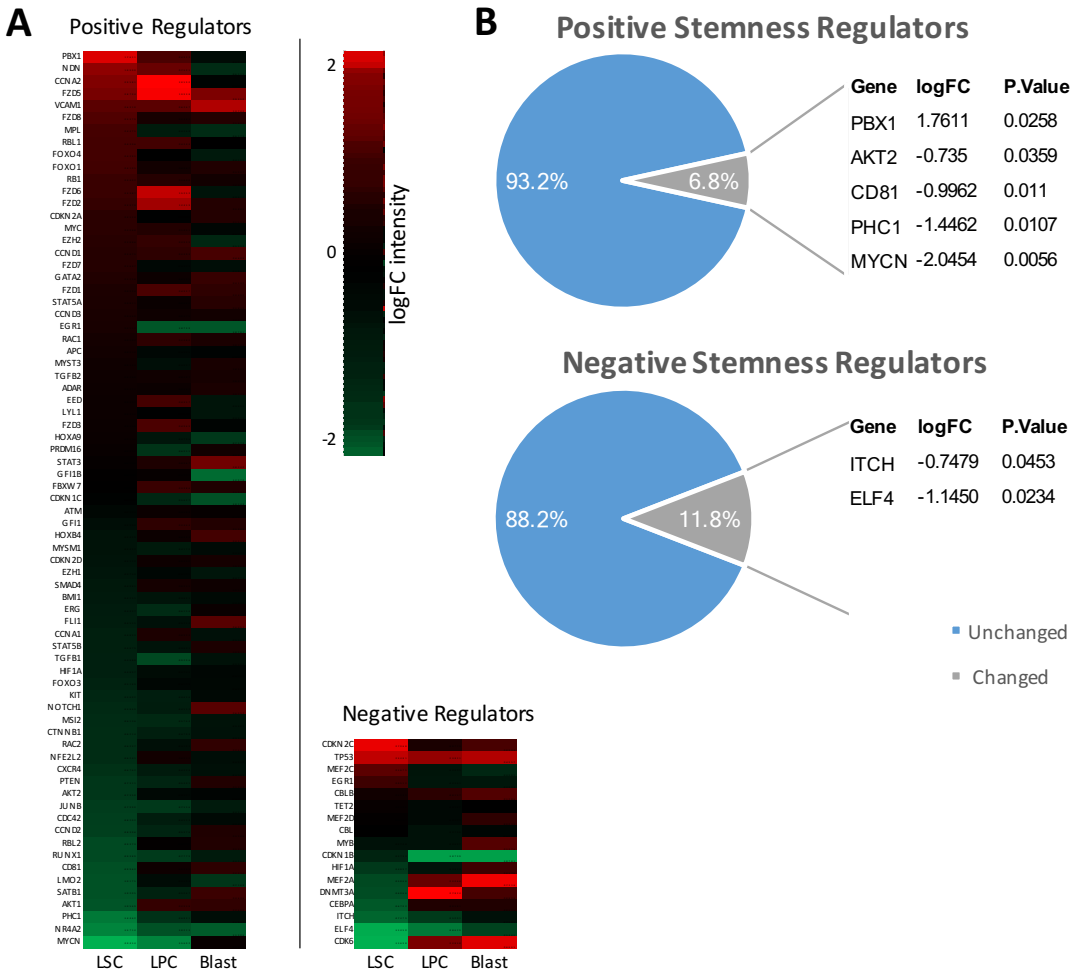




Positive		Negative
Scl1	Necdin	Elf4
Lyl1	JunB	Dnmt3a
Lmo2	ICN	Myb
Gata2	Smad4	Cbl
Runx1	Stat3	CDK6
Erg	Stat5	Itch
Fli-1	HoxB4	Hif1a
Spi1	Cdkn1c	Egr1
Gfi-1	Notch1	Tet2
Gfi1b	Nfe212	Cbl
Evi-1	Satb1	Cbl-b
FoxO1	Mysm1	Cdkn2c
FoxO3	Phc1	INK4a
FoxO4	Frizzled	P27
P21	CXCR4	TP53
Ezh1	VCAM	Cebpa
Bmi1	P57	
Eed	PTEN	
Pbx-1	B-catenin	
Egr-1	Stat3/5	
Mph1	TGFb	
Rae28	c-Kit	
Fbxw7	MPL	
Mycn	Cdc42	
Myc	Adar1	
Nr4a2	Irgm1	
Lkb1	CD81	
Atm1	APC	
Irgm1	Wnt3a	
Myst3	Rac1	
Prdm16	Rac2	
HoxA9	RB1	
Pten	Rbl1	
Ctnnb1	Rbl2	
Akt1/2	Cdkn2a	
Msi2	INK4d	
cMpl	Cyclin A	
Itg14	Cyclin D1	
HIF1a	Cyclin D2	
Wnt3a	Cyclin D3	

<u>Figure 2-3:</u> Patient expression of known genes affecting stemness

(Left panel) Heatmaps of normalized LSC gene expression (log2 intensity) per patient in our D0 and D14 treatment cohorts. Each column represents expression intensity for a single patient. Genes with known positive and negative influence on stemness represent each row of the heatmap and are further depicted in the table (right panel).



<u>Figure 2-4:</u> Altered stemness regulators in AML patients

(A) Log fold-change gene expression intensity from LSC (CD34+CD38-CD90-), LPC (CD34+CD38+), and blast (CD34+) blast cell populations is shown for many known positive and negative stemness regulators. (B) Representation of positive and negative stemness regulators that are changed (P<0.05, grey) and unchanged (P<0.05, blue) in patient LSCs following induction therapy. Significantly altered genes (P<0.05) and their associated log fold-change are depicted.

Gene expression changes are predictive of changes in chemosensitivity

LSCs are thought to adopt multiple means of evading chemotherapy (Warner et al., 2004). To further interrogate these processes in the context of chemotherapy, we selected a panel of 51 candidate genes for validation based on fold-change in LSCs after induction therapy (**Table 2-2**). Genes in our panel are known to affect a variety of biological processes, including: Inflammation, cell cycle regulation, and leukemogenesis. Utilizing an *in vitro* cytotoxicity assay paired with shRNA-mediated knockdown of our panel of candidate genes we see increased chemosensitivity in response to DNR and/or Ara-C (**Figure 2-5 A, Figure 2-6 A, B**). We also observed a decrease in the kinetic of Molm-13 cell growth in the presence of 5-LO knockdown, suggesting that 5-LO may not only media leukemic cell chemoresistance but also proliferative capacity. Top hits from our cytotoxicity assay based on percent reduction of leukemic cells included: 5-LO, CST7, S100A8, S100A9, LYZ, and CD36, with knockdown of these genes also increasing caspase-mediated apoptosis (**Figure 2-5 B, C**). Consistent with our AML patient data, treatment of human AML cell lines with either DNR or Ara-C in vitro resulted in an induction of 5-LO, CD36 and CST7 mRNA in Molm-13 cells (**Figure 2-7 A-C**). These data demonstrate that leukemic cell response to chemotherapy is coordinated by multiple genes with disparate biological functions.

As we observed a large amount of variability in baseline expression of 5-LO, CST7, and CD36 (**Figure 2-7 D-F**), we next sought to assess whether either high baseline gene expression correlated with relative chemosensitivity of these leukemic cell lines. We therefore treated a panel of 10 human AML cell lines with either Ara-

Table 2-2: Putative chemoresistance genes

Gene	logFC	P-value	Gene	logFC	P-value
S100A8	4.61	0.0000	CTSO	-1.03	0.0283
RRM2	3.71	0.0026	PAK2	-1.06	0.0006
CLU	3.54	0.0003	PIK3CA	-1.12	0.0476
VCAN	3.07	0.0019	MIB1	-1.19	0.0351
CFH	2.95	0.0029	ETS2	-1.21	0.0194
ALOX5	2.88	0.0131	SKIL	-1.28	0.0200
LYZ	2.59	0.0002	RICTOR	-1.28	0.0022
S100A9	2.57	0.0003	ETV3	-1.33	0.0015
PIM1	2.12	0.0007	SOD2	-1.34	0.0354
CD36	2.02	0.0155	RBL2	-1.44	0.0102
CST7	1.95	0.0162	TXNDC12	-1.46	0.0470
HLF	1.91	0.0381	USP3	-1.48	0.0412
ETS1	1.74	0.0233	PRKCI	-1.57	0.0269
TCF4	1.68	0.0312	IFNGR1	-1.59	0.0072
INHBC	1.68	0.0239	RASGEF1A	-1.65	0.0446
FKBP5	1.35	0.0047	IL23A	-1.68	0.0134
RAD51	1.35	0.0190	ETV6	-1.68	0.0135
FBXW11	1.09	0.0275	PDK1	-1.74	0.0018
HNRNPD	0.76	0.0247	FOSL2	-1.83	0.0450
SIRPA	0.64	0.0408	S EPT6	-2.01	0.0017
CRB1	0.47	0.0568	ANGPT1	-2.09	0.0041
HDAC11	-0.57	0.0348	SMAD2	-2.10	0.0190
IL6ST	-0.78	0.0366	TGFBR1	-2.47	0.0262
ALDH6A1	-0.88	0.0491	IKBKB	-2.91	0.0042
FGFR1OP2	-0.90	0.0043	MGEA5	-3.27	0.0000
RAPGEF1	-1.01	0.0438			

Panel of putative chemoresistance mediators with their associated log fold-change and *P*-value observed in patient LSCs following induction therapy.

Figure 2-5: Validation of putative chemoresistance mediators

(A) Change in human AML cell line chemosensitivity (PI- cell count) to Ara-C and DNR in the presence of shRNA-mediated knockdown of putative chemoresistance genes that were upregulated in patient LSCs. (B) Change in human AML cell line Ara-C or DNR chemosensitivity (PI- cell count) in the presence of multiple shRNAs targeted to top hits from initial validation panel. (C) Measurement of apoptosis as determined by percentage caspase-3-cleavage positive cells. Molm-13 cells contained either 5-LO (left panel) or CST7 (right panel) knockdown and were exposed to DNR or Ara-C, respectively. The results are presented as mean ± standard error mean (SEM). P-values were determined by the Student's t-test; *P < 0.05, **P < 0.01, ***P < 0.001.

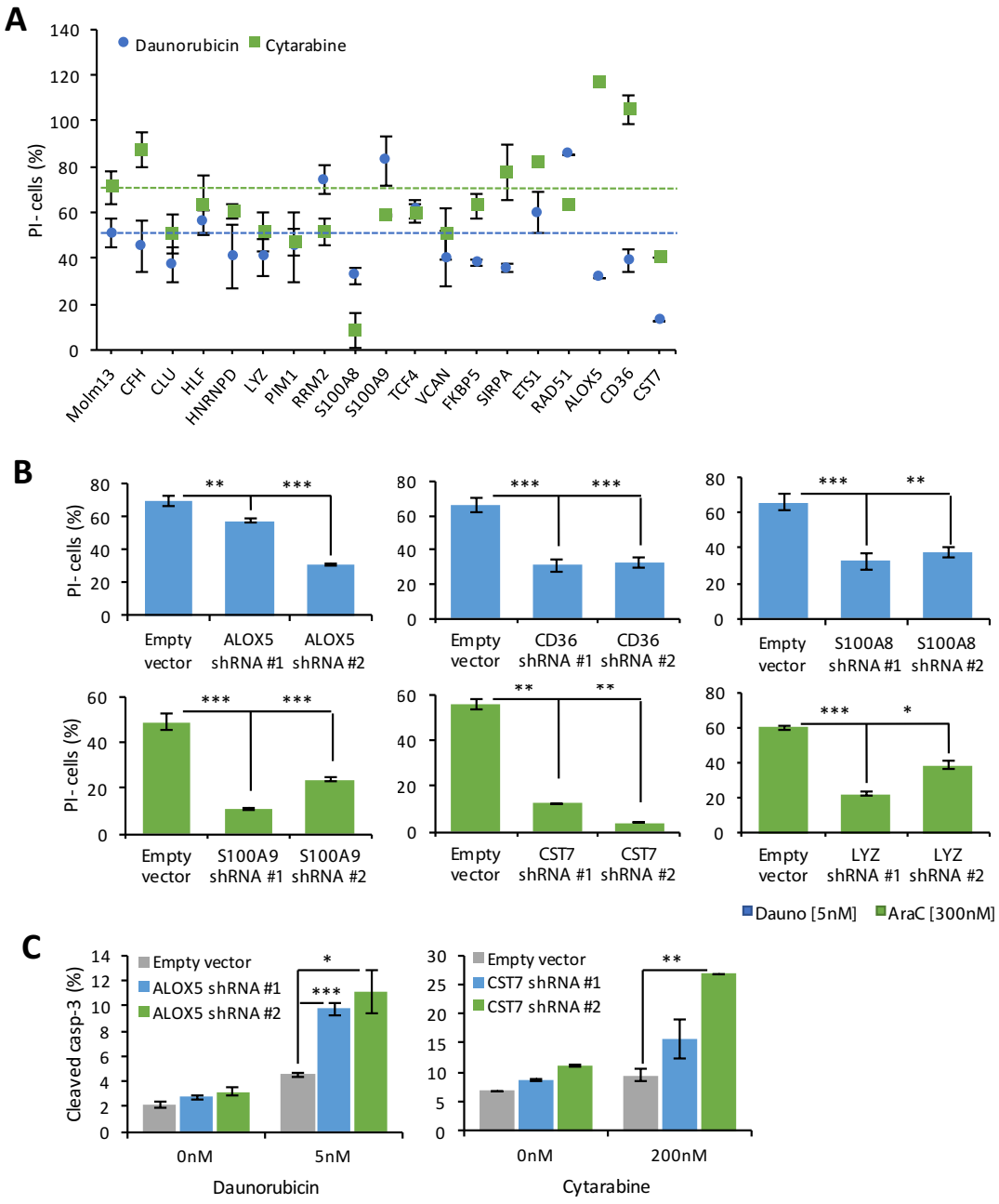
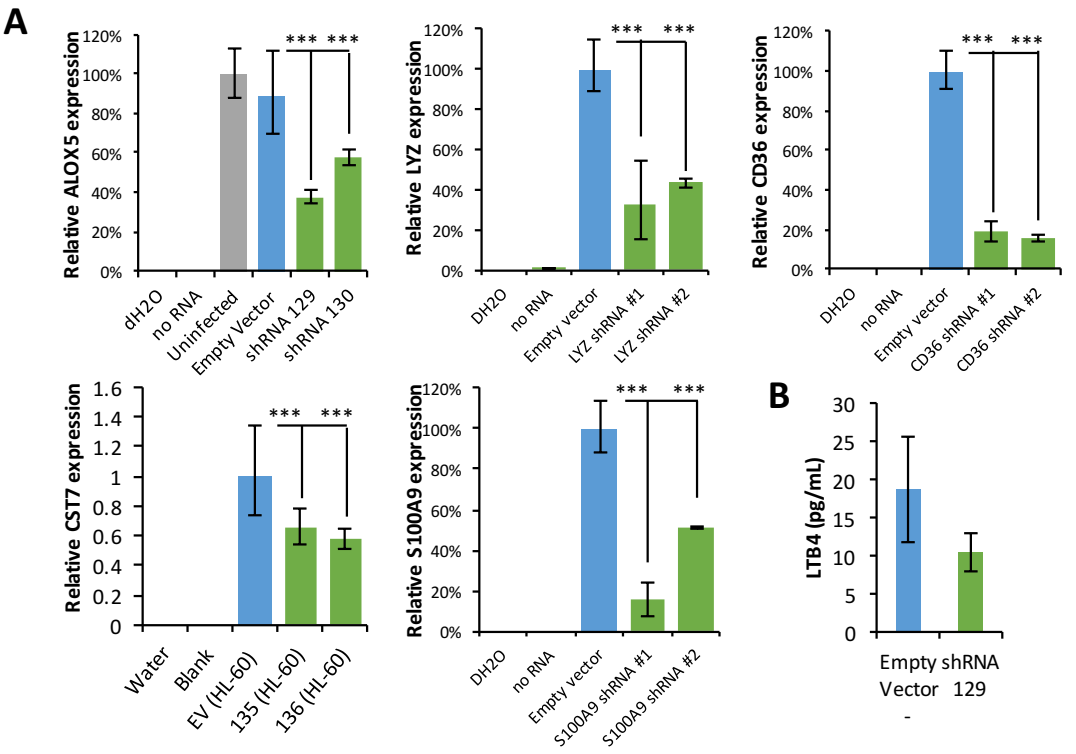
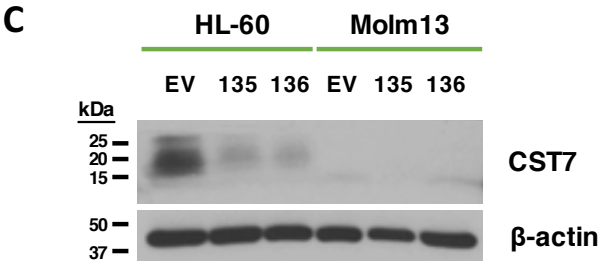

A
Daunorubicin
Cytarabine
PI- cells (%)
Molm13
CFH
CLU
HLF
HNRNPD
LYZ
PIM1
RRM2
S100A8
S100A9
TCF4
VCAN
FKBP5
SIRPA
ETS1
RAD51
ALOX5
CD36
CST7
B
PI- cells (%)
Empty vector
ALOX5 shRNA #1
ALOX5 shRNA #2
CD36 shRNA #1
CD36 shRNA #2
S100A8 shRNA #1
S100A8 shRNA #2
S100A9 shRNA #1
S100A9 shRNA #2
CST7 shRNA #1
CST7 shRNA #2
LYZ shRNA #1
LYZ shRNA #2
Dauno [5nM]
AraC [300nM]
C
Cleaved casp-3 (%)
Empty vector
ALOX5 shRNA #1
ALOX5 shRNA #2
CST7 shRNA #1
CST7 shRNA #2
0nM
5nM
200nM
Daunorubicin
Cytarabine

Figure 2-6: shRNA knockdown verification and analysis of cell growth defects

(A) Quantitation of shRNA-mediated knockdown of putative chemoresistance genes as determined by qPCR. (B) Levels of LTB4 secretion in the presence of 5-LO knockdown as measured by ELISA. (C) Western blot of CST7 protein expression in either HL-60 or Molm-13 cells in the presence of knockdown (shRNAs 135, 136). (D) Kinetic of Molm-13 cell growth in the presence of 5-LO shRNA-mediated knockdown. The results are presented as mean ± standard error mean (SEM). P-values were determined by the Student's t-test; *P < 0.05, **P < 0.01, ***P < 0.001.





		Intensity (avg)	Intensity over bkg	Normalized to EV	%
	Bkg	185.859			
HL-60	EV	100.213	85.646	1	**100%**
	135	163.145	22.714	0.265	**27%**
	136	170.128	15.731	0.183	**18%**
Molm13	EV	185.689	0.17	0.001	**0%**
	135	185.137	0.722	0.008	**1%**
	136	183.727	2.132	0.024	**2%**

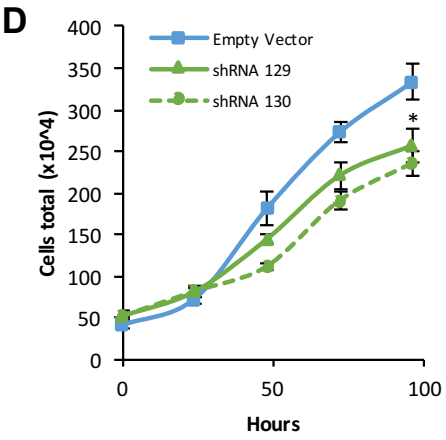
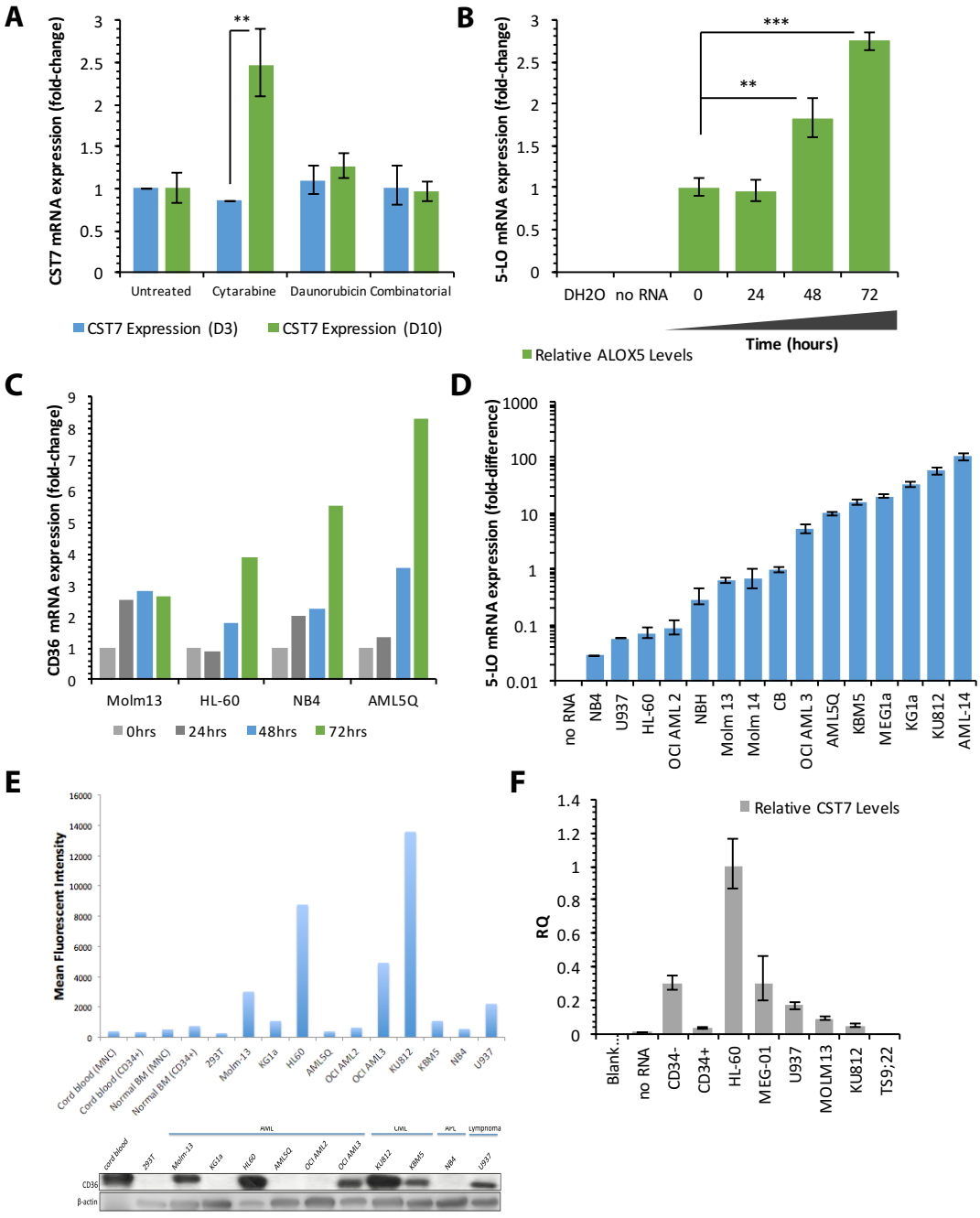


Figure 2-7: Baseline and induced chemoresistance gene expression

(A) Fold-change induction of CST7 expression after 3 days (D3) or 10 days (D10) in HL-60 cells exposed to either Ara-C, DNR, or both for 72 hours. (B) Fold-change induction of 5-LO expression in Molm-13 cells measured daily. (C) Fold-change induction of CD36 expression in Molm-13, HL-60, NB4, and AML5Q AML cell lines exposed to DNR. Baseline expression of (D) 5-LO, (E) CD36, and (F) CST7 in a panel of leukemic cell lines. All transcript levels were measured by qPCR. The results are presented as mean ± standard error mean (SEM). P-values were determined by the Student's t-test; *P < 0.05, **P < 0.01, ***P < 0.001.


A
CST7 mRNA expression (fold-change)
Untreated
Cytarabine
Daunorubicin
Combinatorial
CST7 Expression (D3)
CST7 Expression (D10)
B
5-LO mRNA expression (fold-change)
DH2O
no RNA
Time (hours)
Relative ALOX5 Levels
C
CD36 mRNA expression (fold-change)
Molm13
HL-60
NB4
AML5Q
0hrs
24hrs
48hrs
72hrs
D
5-LO mRNA expression (fold-difference)
E
Mean Fluorescent Intensity
F
Relative CST7 Levels
RQ

C, DNR, or both drugs together. Cell survival, baseline 5-LO expression, and 5-LO transcript induction after treatment were then measured. While we do not see an apparent relationship between baseline 5-LO expression and chemoresistance (**Figure 2-8 A**), the cell lines that induced the highest levels of 5-LO were also the most chemoresistant (**Figure 2-8 B**). Interestingly, we see that cell lines that induce 5-LO in response to DNR treatment, are more resistance to Ara-C, however the inverse relationship was not tested. These observations are in agreement with our hypothesis that improved ability to resist chemotherapy is primarily a result of capacity to induce expression of chemoresistance genes. Together, these studies identify bona fide mediators of chemoresistance in AML that represent a broad spectrum of biological functions.

DISCUSSION

Here, we developed a platform for identifying and validating putative chemoresistance mediators in the most clinically-relevant cell populations in patients undergoing chemotherapy. The hypothesis tested was that these dysregulated genes will provide tractable targets for combinatorial inhibition with induction therapy. We found that several genes upregulated in our AML patient samples, including 5-LO, CD36, CST7, and S100A9, improved chemosensitivity to cytarabine and daunorubicin when knocked down *in vitro*. This study provides a basis for identification of additional chemoresistance mediators and offers guidance for clinical development of therapies in combination with standard-of-care induction therapy.

As cytarabine and daunorubicin primarily target dividing cells, the quiescent nature of the LSC is thought to be one mechanism of chemotherapy evasion

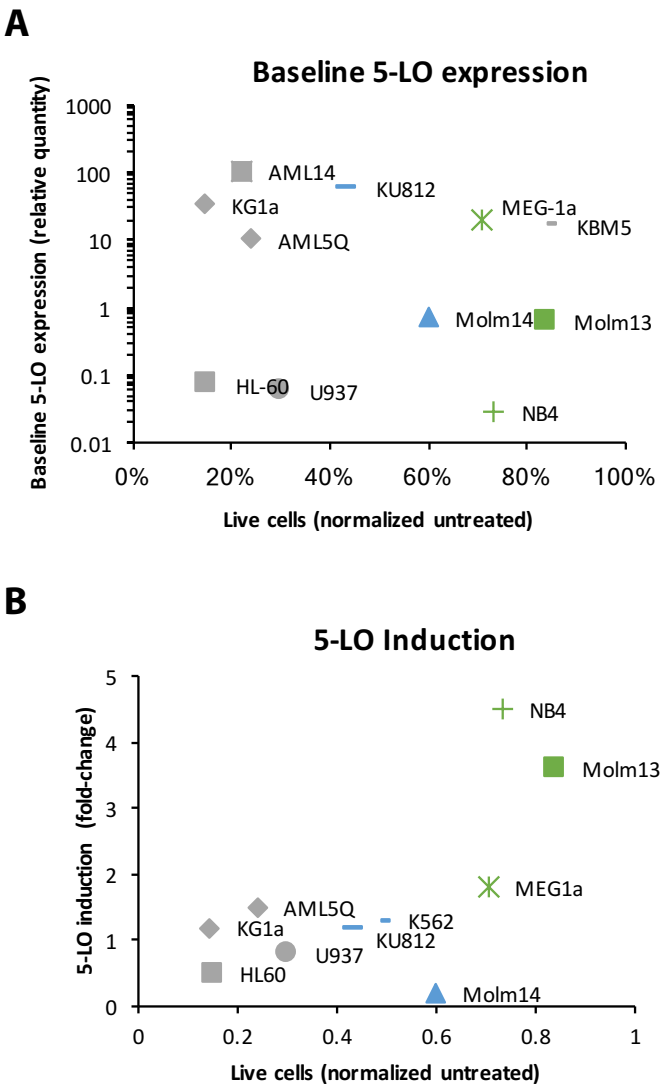


Figure 2-8: Level of 5-LO induction predicts chemosensitivity

Leukemic cell line viability (PI- cell count) upon treatment with Ara-C as a function of (A) baseline 5-LO expression or (B) level of 5-LO induction upon exposure to DNR for 72 hours as measured by qPCR.

(Dean et al., 2005; Warner et al., 2004). Interestingly, GSEA of our dysregulated LSC gene set showed strong enrichment of pathways promoting self-renewal and proliferation following chemotherapy. Furthermore, we see that the majority of known stemness regulators remain unchanged in our LSC gene set. This suggests that, while LSCs maintain their stem cell capacity, they are primed for expansion immediately following induction therapy. It is unclear whether this relates to chemotherapy evasion or is a reflection of general cell expansion following myelodepletion of the bone marrow after chemotherapy (Woelich et al., 2017). However, through our validation studies we see that reduced 5-LO expression slows the kinetic of leukemic cell growth and also decreases chemoresistance. This suggests that at least some genes identified by our studies may control both leukemic cell growth and resistance to therapy.

Several other putative chemoresistance genes that regulate drug efflux and apoptosis have been investigated previously (Callaghan et al., 2014). Most notably was the development of MRP1 and p-glycoprotein inhibitors that ultimately failed to exhibit clinical efficacy, despite significant positive *in vitro* data (Peng et al., 2004; Rumpold et al., 2007). While the involvement of drug efflux pumps in chemoresistance is likely one way by which cells evade chemotherapy, these studies demonstrated that there is significant overlap in chemoresistance processes and that early evidence in primary patient samples is crucial for future clinical development of targeted therapies. To our knowledge, our study is the first to use paired primary AML cases to assess gene expression changes *in vivo* for the identification of novel chemoresistance mediators. Moreover, our study looks in specific cell populations separated by rigorous immunophenotyping (Horton & Huntly, 2012). This represents an unbiased approach based on direct findings from patients undergoing therapy.

Through our studies, we have identified genes involved in a variety of biological process, beyond the mechanisms of drug efflux and cell-cycle regulation, including: Inflammation, lipid metabolism, and protein turnover. These pathways remain relatively understudied in relation to chemoresistance. 5-LO, a gene with canonical roles in inflammation, appears to confer chemoresistance through an anti-apoptotic mechanism, however additional study of its mechanism of action is necessary (and is further described in **Chapter Three**). Leukotrienes, the biological product of 5-LO activity have been shown to control inflammation in both normal and malignant contexts, partially through involvement of JAK-STAT signaling (Y. Chen et al., 2016; D. Wang & Dubois, 2010). Our KEGG pathway analysis of dysregulated LSC genes shows enrichment of cytokine-cytokine receptor signaling and JAK-STAT signaling, providing additional support for the role of inflammation and cell proliferation in LSC survival. Further investigation of leukotrienes and their control of inflammation in the context of chemotherapeutic insult is therefore required.

CHAPTER THREE:
CHARACTERIZATION OF 5-LIPOXYGENASE IN LEUKEMIC STEM CELL CHEMOTHERAPY EVASION

INTRODUCTION

Acute myeloid leukemia (AML) represents the expansion of immature myeloid cells in the bone marrow following the accumulation of numerous genetic and epigenetic changes (Renneville et al., 2008). AML is thought to arise from, and be maintained by, a small population of leukemic stem cells (LSCs) that possess the ability to self-renew (Bonnet & Dick, 1997). Due to the non-specific nature of chemotherapy, normal hematopoiesis is also further compromised with treatment, resulting in severe comorbidities, particularly in the elderly (Büchner et al., 2009; Ossenkoppele & Lowenberg, 2015). Because LSCs are relatively therapy-resistant and can re-initiate disease following therapy, development of novel strategies to sensitize LSCs to chemotherapy hold the promise of improving outcomes while also avoiding the negative consequences of myelosuppression resulting from chemotherapy (Ravandi & Estrov, 2006).

Standard induction therapy is comprised of a combination of the nucleoside analog cytarabine (Ara-C) and an anthracycline such as daunorubicin (DNR) (Ogbomo et al., 2008; Quigley et al., 1980; Rowe & Tallman, 2010). This treatment strategy has essentially remained unchanged for thirty years and while the majority of patients achieve complete remission following induction therapy, most will relapse without maintenance therapy or bone marrow transplantation (Burnett et al., 2009; Roboz, 2011). Finding new ways by which to bolster the efficacy of first line treatments, particularly against LSCs, is therefore imperative.

LSCs are thought to be relatively quiescent, which renders them less responsive to chemotherapy (Warner et al., 2004). Multiple additional mechanisms of chemoresistance have been identified in AML, including genes that regulate drug efflux and apoptosis, such as MRP1 and BCL-2 (Callaghan et al., 2014; Dean et al., 2005). However, high expression of ABC-family transporters are present in both LSC and hematopoietic stem cell (HSC) populations, with inhibitors of MRP1 and P-gp ultimately failing to exhibit clinical efficacy (Peng et al., 2004; Ravandi & Estrov, 2006; Rumpold et al., 2007). Several other cellular processes, including modulation of inflammation, lipid metabolism, and epigenetic modification have also recently been linked to chemoresistance (de Visser & Jonkers, 2009; Guryanova et al., 2016; H. Ye et al., 2016), but it is not currently clear how to translate these findings to the clinic. Collectively, these studies underscore that chemoresistance likely involves multiple pathways, yet the specificity of these processes with respect to LSC response to chemotherapy is currently unknown. Thus, understanding LSC mechanisms of resistance represents an important unmet need in AML biology.

Herein we show that 5-lipoxygenase (5-LO), a protein with canonical roles in inflammation, mediates AML chemoresistance through a leukotriene-dependent

process in AML blasts. As roles for 5-LO and other inflammatory mediators have not been extensively studied in the context of leukemic cell chemoresistance, and prior studies have implicated 5-LO in the self-renewal of chronic phase CML stem cells through unclear mechanisms (Y. Chen, Hu, et al., 2009), we investigated the role of 5-LO in AML chemoresistance. Genetic and pharmacologic loss-of-function experiments confirm that 5-LO not only positively regulated the leukemogenic potential of LSCs but mediated their chemosensitivity *in vitro* and *in vivo*. Importantly, 5-LO knockout (*Alox5*$^{-/-}$) HSCs exhibit enhanced self-renewal capacity as demonstrated by long-term reconstitution potential in serial transplantation, suggesting that targeted inhibition of 5-LO may potentially enhance normal HSC function while simultaneously improving elimination of LSCs. Together, these studies highlight combinatorial 5-LO inhibition with standard induction chemotherapy as a tractable therapeutic avenue for improving treatment responses in AML.

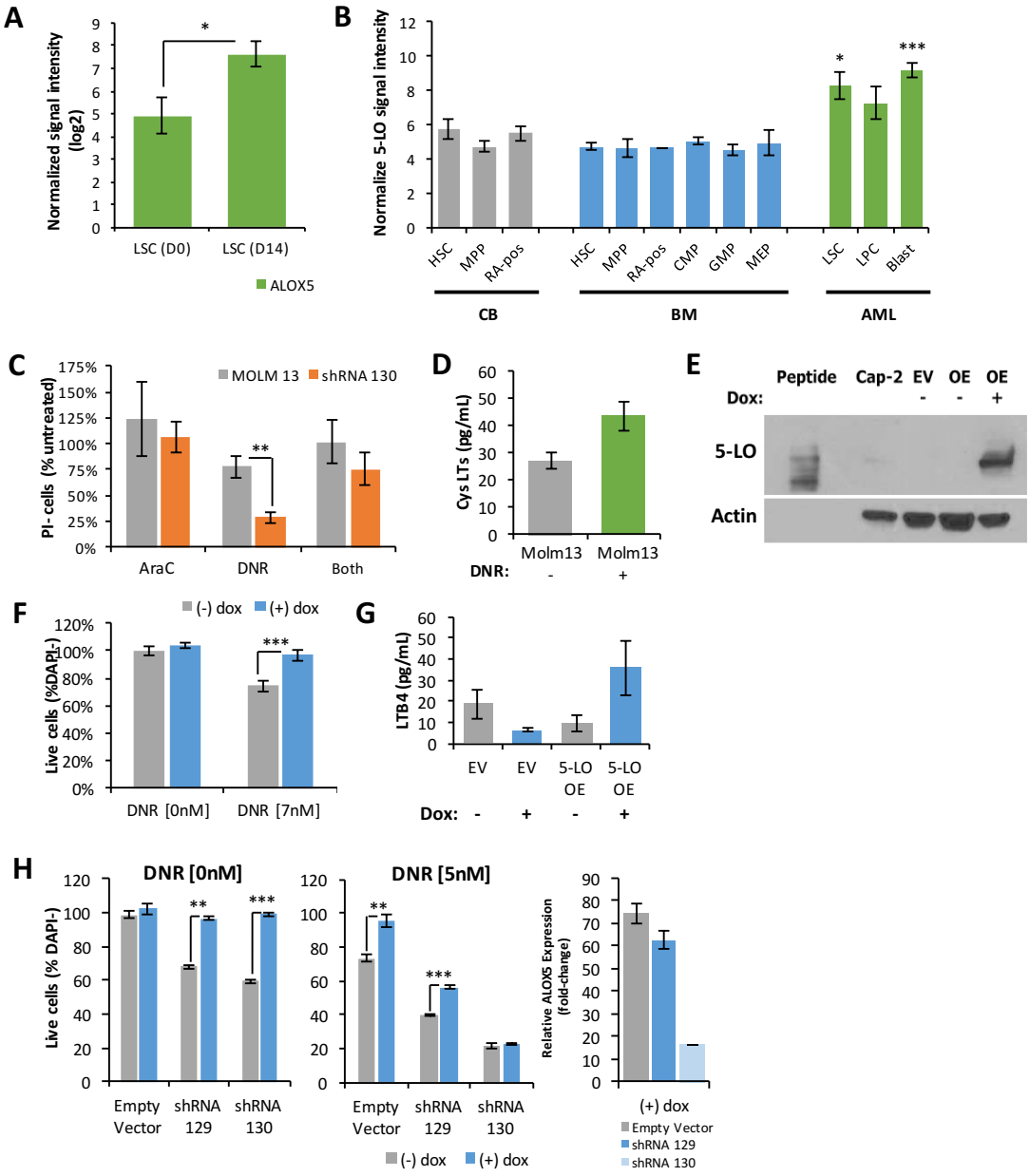
RESULTS

5-LO is induced in response to DNR and promotes therapy resistance in AML

We have identified several genes upregulated in paired diagnostic:post-induction therapy AML bone marrow (BM) samples from patients with residual disease after induction chemotherapy. Among these 5-LO exhibited a log-fold induction of 2.8763 (*P*-value: 0.0131) specifically in patient LSC-enriched ($CD34^{-}$ $CD38^{+}CD90^{-}$) cells (**Figure 3-1 A**). Out of 3205 differentially expressed LSC genes ($P < 0.05$), 5-LO ranked in the top 0.22% by fold-change intensity. 5-LO was

<u>Figure 3-1:</u> 5-LO is induced with DNR treatment and overexpression increases chemoresistance

(A) Average $\log_2$ probe intensity for patient LSCs immediately before (D0) or 14 days following (D14) induction therapy (B) GEXC average 5-LO probe intensity for a panel of normal and AML cell types. (C) Measurement of live cells by PI staining in uninfected and 5-LO shRNA-containing Molm-13 cells. Cells were exposed to either cytarabine, DNR, or both in combination for 72 hours. (D) Cysteinyl leukotriene synthesis as measured by ELISA in Molm-13 cells treated with DNR. (E) Western blot of 5-LO expression levels with doxycycline addition (OE+). Purified 5-LO peptide and Capan-2 cells were used as positive controls for detection of 5-LO, while empty vector (EV) and uninduced overexpression-containing cell lysates (OE-) were negative controls. (F) 5-LO overexpression induced by addition of doxycycline in Molm-13 cells treated with DNR. Relative live cells after treatment were measured as a percentage of %DAPI- cells. (G) ELISA for leukotriene B4 synthesis with 5-LO overexpression. (H) %DAPI- live cell frequency with 5-LO overexpression in the presence of two separate shRNAs (129, 130) against 5-LO in either the absence of DNR (left panel) or presence of DNR (right panel), and associated 5-LO expression as measured by qPCR. The results are presented as mean ± standard error mean (SEM). *P*-values were determined by the Student's t-test; $^{**}P < 0.01$, $^{***}P < 0.001$.

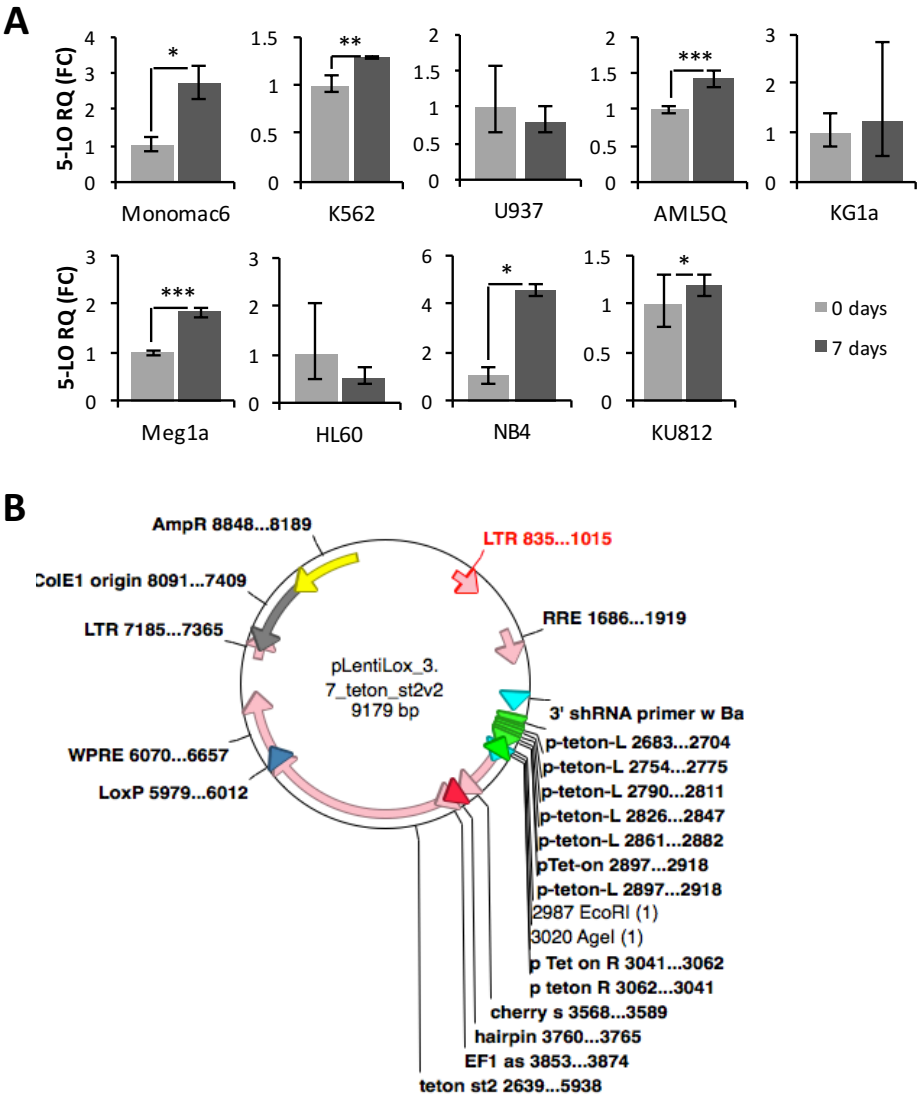

A
Normalized signal intensity (log2)
LSC (D0)
LSC (D14)
ALOX5
B
Normalize 5-LO signal intensity
HSC
MPP
RA-pos
CMP
GMP
MEP
LSC
LPC
Blast
CB
BM
AML
C
PI- cells (% untreated)
MOLM 13
shRNA 130
AraC
DNR
Both
D
Cys LTs (pg/mL)
Molm13
DNR:
E
Peptide
Cap-2
EV
OE
Dox:
5-LO
Actin
F
(-) dox
(+) dox
Live cells (%DAPI-)
DNR [0nM]
DNR [7nM]
G
LTB4 (pg/mL)
EV
5-LO OE
Dox:
H
DNR [0nM]
DNR [5nM]
Live cells (% DAPI-)
Empty Vector
shRNA 129
shRNA 130
Relative ALOX5 Expression (fold-change)
(+) dox

expressed at significantly higher levels in leukemic cell types, including LSCs and LPCs when compared to normal BM and cord blood (CB) hematopoietic stem and progenitor cells (HSPCs) (**Figure 3-1 B**) (Gentles et al., 2012; Seita et al., 2012). Consistent with our observations in AML patient bone marrow, 5-LO was significantly induced in several human leukemic blast cell lines treated with DNR *in vitro* (**Figure 3-2 A**). 5-LO knockdown resulted in higher sensitivity of AML cells to DNR, suggesting that 5-LO primarily mediates blasts responses to DNR (**Figure 3-1 C**).

5-LO facilitates a required step in the synthesis of LTB_4 and cysteinyl leukotrienes, and has been shown to promote cancer cell proliferation and migration in several solid tumor models (D. Wang & Dubois, 2010). AML LSCs exhibit high expression of receptors for both LTB_4 and cysteinyl leukotrienes, as well as elevated 5-LO expression compared to normal hematopoietic stem and progenitor cells (Gentles et al., 2012; Seita et al., 2012; D. Wang & Dubois, 2010). A high level of LTB_4 induction following imatinib therapy is associated with poor response rates in patients with CML (Lucas et al., 2014). Thus, we sought to determine whether leukotriene synthesis is also induced with chemotherapy in AML. Heightened levels of cysteinyl leukotriene production were observed upon treatment of the human AML cell line Molm-13 with DNR (**Figure 3-1 D**). Overexpression of 5-LO cDNA in Molm-13 cells reduced the cytotoxic effect of DNR, completely abrogating DNR-induced cell death (**Figure 3-1 E, F, Figure 3-2 B**). Overexpression of 5-LO also resulted in increased synthesis of LTB_4, confirming that exogenous 5-LO induces the same biological activity as endogenous 5-LO (**Figure 3-1 G**). While constitutive shRNA-mediated 5-LO knockdown increased leukemic cell sensitivity to DNR treatment, overexpression of 5-LO in these cells rescued both the observed cell growth

<u>Figure 3-2:</u> 5-LO leukemic cell line induction and overexpression

(A) Fold-change (FC) in relative transcript quantity (RQ) of 5-LO in a panel of human leukemia cell lines. Number of days refers to length of DNR exposure. (B) Plasmid features and insertion site of full-length 5-LO cDNA in the doxycycline-inducible pLentiLox expression vector. (C) Cell cycle distribution determined by Ki67 staining in MLL-AF9 leukemic cells treated with leukotrienes either alone or in combination. (D) LTB4 synthesis as measured by ELISA in vehicle and Zileuton treated Molm-13 cells. The results are presented as mean ± standard error mean (SEM). *P*-values were determined by the Student's t-test; **P* < 0.05. ***P* < 0.01.


A
5-LO RQ (FC)
Monomac6
K562
U937
AML5Q
KG1a
Meg1a
HL60
NB4
KU812
0 days
7 days
B
AmpR 8848...8189
LTR 835...1015
ColE1 origin 8091...7409
RRE 1686...1919
LTR 7185...7365
pLentiLox_3.
7_teton_st2v2
9179 bp
3' shRNA primer w Ba
p-teton-L 2683...2704
p-teton-L 2754...2775
p-teton-L 2790...2811
p-teton-L 2826...2847
p-teton-L 2861...2882
pTet-on 2897...2918
p-teton-L 2897...2918
2987 EcoRI (1)
3020 AgeI (1)
p Tet on R 3041...3062
p teton R 3062...3041
cherry s 3568...3589
hairpin 3760...3765
EF1 as 3853...3874
teton st2 2639...5938
WPRE 6070...6657
LoxP 5979...6012
C
G2M
S
G1
G0
% GFP+ cells
Com...
B4
C4
D4
E4
Leukotriene [100uM]
D
LTB4 (pg/mL)
Vehicle
Zileuton

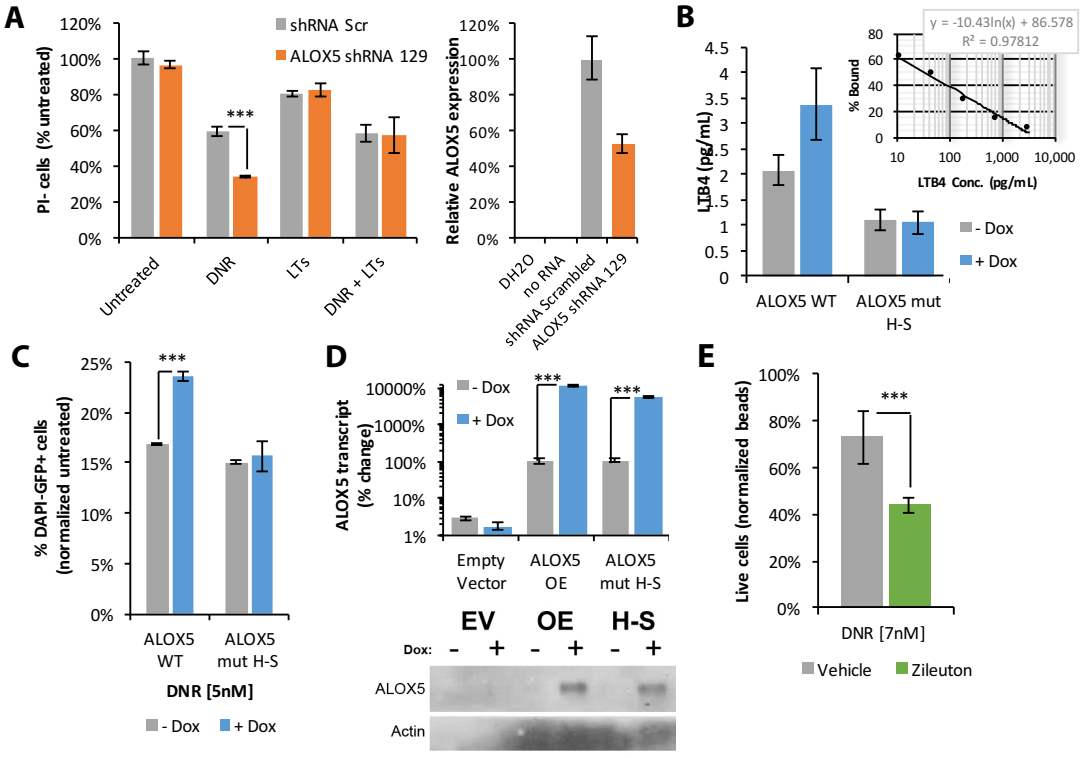
defect and chemosensitizing effect of 5-LO knockdown (**Figure 3-1 H**). Together, these data provide support of induced 5-LO expression as a DNR-specific mediator of resistance in AML.

Leukotrienes mediate 5-LO dependent therapy resistance

As DNR treatment induces 5-LO, as well as cysteinyl leukotrienes, we tested whether leukotrienes mediate its chemoprotective effect. Consistent with this model, administration of purified leukotrienes (LTB_4, LTC_4, LTD_4, and LTE_4) completely rescued the chemosensitizing effect of 5-LO knockdown in Molm-13 cells (**Figure 3-3 A**). Addition of leukotrienes to primary mouse MLL-AF9 blasts *ex vivo* promoted an increased percentage of cells in G1, with a concomitant decrease in S-phase percentage as assessed by Ki67 staining (**Figure 3-2 C**). To directly test whether the observed increase in chemoresistance with 5-LO overexpression is due to increased presence of the 5-LO protein or its leukotriene-synthetic capacity, a catalytically-inactivated form of 5-LO was expressed which is deficient in leukotriene synthesis (**Figure 3-3 B**). While induced overexpression of wild-type (WT) 5-LO results in an increase in chemoresistance (**Figure 3-1 E, Figure 3-3 C**), expression of catalytically-inactive 5-LO did not result in an observable change in chemoresistance despite comparable levels of overexpression (**Figure 3-3 C, D**). Pharmacological inhibition of 5-LO using Zileuton in combination with DNR resulted in a 40% reduction in LTB_4 synthesis and increased the chemosensitivity of Molm-13 cells to DNR (**Figure 3-3 E, Figure 3-2 D**). This is in agreement with the increased DNR sensitivity following 5-LO depletion (**Figure 3-1 C**). Together, these data implicate 5-LO, and specifically its leukotriene synthetic capacity, as a chemoresistance pathway in AML.

Figure 3-3: Leukotrienes mediate chemoresistance

(A) PI- Molm-13 cells containing scrambled (scr) or 5-LO knockdown (shRNA 129) normalized to untreated. Cells were treated with DNR and a mixture of leukotrienes (LTB4, LTC4, LTD4, and LTE4), or DNR and leukotrienes alone (left panel) and relative 5-LO expression determined by qPCR (right panel). (B) LTB4 synthesis as determined by ELISA in Molm-13 cells induced with doxycycline to overexpress either WT 5-LO (*Alox5* WT) or catalytically-inactivated 5-LO (*Alox5* mut H-S). (C) DNR treated Molm-13 cells induced with doxycycline to overexpress either WT 5-LO (*Alox5* WT) or catalytically-inactivated 5-LO (*Alox5* mut H-S). Live cells were measured as %DAPI-, which was normalized to untreated. (D) Level of overexpression of WT 5-LO or catalytically-inactivated 5-LO as measured by qPCR (top panel) and western blot (bottom panel). (E) PI- Molm-13 cells after treatment with either Zileuton or vehicle (DMSO) at increasing concentrations in the presence of DNR. The results are presented as mean ± standard error mean (SEM). *P*-values were determined by the Student's t-test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.


A
PI- cells (% untreated)
shRNA Scr
ALOX5 shRNA 129
***
Untreated
DNR
LTs
DNR + LTs
Relative ALOX5 expression
DH2O
no RNA
shRNA Scrambled
ALOX5 shRNA 129
B
LTB4 (pg/mL)
y = -10.43ln(x) + 86.578
R² = 0.97812
% Bound
LTB4 Conc. (pg/mL)
- Dox
+ Dox
ALOX5 WT
ALOX5 mut H-S
C
% DAPI-GFP+ cells (normalized untreated)
***
ALOX5 WT
ALOX5 mut H-S
DNR [5nM]
- Dox
+ Dox
D
ALOX5 transcript (% change)
- Dox
+ Dox
***
***
Empty Vector
ALOX5 OE
ALOX5 mut H-S
EV
OE
H-S
Dox: - + - + - +
ALOX5
Actin
E
Live cells (normalized beads)
***
DNR [7nM]
Vehicle
Zileuton

Alox5$^{-/-}$ leukemic blasts transcriptional programs and response to DNR

To investigate mechanisms of 5-LO regulation of chemoresponses, gene expression changes in MLL-AF9 WT and Alox5-/- leukemias were measured following exposure to DNR. The MLL-AF9 leukemic model was adopted as this gene fusion has been shown to result in a particularly aggressive form of leukemia in patients (Stavropoulou et al., 2016). *Alox5*$^{-/-}$ AMLs were generated by retrovirally transducing *Alox5*$^{-/-}$ bone marrow HSPCs (LSK; Lin^{-}Sca-1^{+}c-Kit^{+}) with the MLL-AF9 fusion, as previously published (Krivtsov et al., 2006). MLL-AF9 WT and *Alox5*$^{-/-}$ blasts were then exposed to DNR *ex vivo*. RNA sequencing revealed distinct responses of WT and KO blasts (**Figure 3-4 A**). Untreated WT and *Alox5*$^{-/-}$ leukemias exhibit differential expression of 5840 genes, while there were 4337 differentially expressed genes between treated WT and *Alox5*$^{-/-}$ leukemias (P-value < 0.05), with a 31.7% overlap between treated and untreated samples (**Figure 3-5 A, Figure 3-4 B**), suggesting that a significant subset of genes between WT and *Alox5*$^{-/-}$ blasts are responsive to chemotherapy (**Figure 3-4 B**).

To determine the potential of 5-LO in mediating the observed resistance phenotype through interaction with other known resistance mediators, differences in expression of these genes pre- and post-therapy were evaluated. Indeed, several putative chemoresistance genes identified by our studies, including S100a8, S100a9, and Lyz2 were significantly upregulated in WT but not *Alox5*$^{-/-}$ leukemias, suggesting an interdependency between these genes and 5-LO (**Figure 3-5 B**). Several known chemoresistance mediators were also upregulated in WT but not *Alox5*$^{-/-}$, including: Cd33, Mdm2, and c-Kit (**Figure 3-5 B**). A number of genes controlling DNA

Figure 3-4: 5-LO leukemic cell gene expression differs from WT

(A) Multidimensional scaling (left panel) and hierarchical clustering (right panel) of WT and *Alox5*$^{-/-}$ leukemic cell gene expression with or without DNR treatment. (B) Venn diagram of differential gene expression overlap between untreated WT and *Alox5*$^{-/-}$ leukemias (WT[0] vs KO[0]), DNR treated WT and *Alox5*$^{-/-}$ leukemias (WT[5] vs KO[5]), and untreated and treated *Alox5*$^{-/-}$ leukemias (KO[0] vs KO[5]). Total differentially regulated genes for each treatment group are indicated adjacent to each circle. Total number of overlapping genes is depicted within each section of the diagram. (C) Enrichment plots from GSEA of pathways and targets upregulated (positive enrichment score) in WT vs *Alox5*$^{-/-}$ leukemic cells. (D, E) Spectral shift of Hoechst 33342 peak emission with increasing concentration of DNR in WT and *Alox5*$^{-/-}$ MLL-AF9 leukemic cells treated *ex vivo*. The results are presented as mean ± standard error mean (SEM). *P*-values were determined by the Student's t-test; $*P < 0.05$.

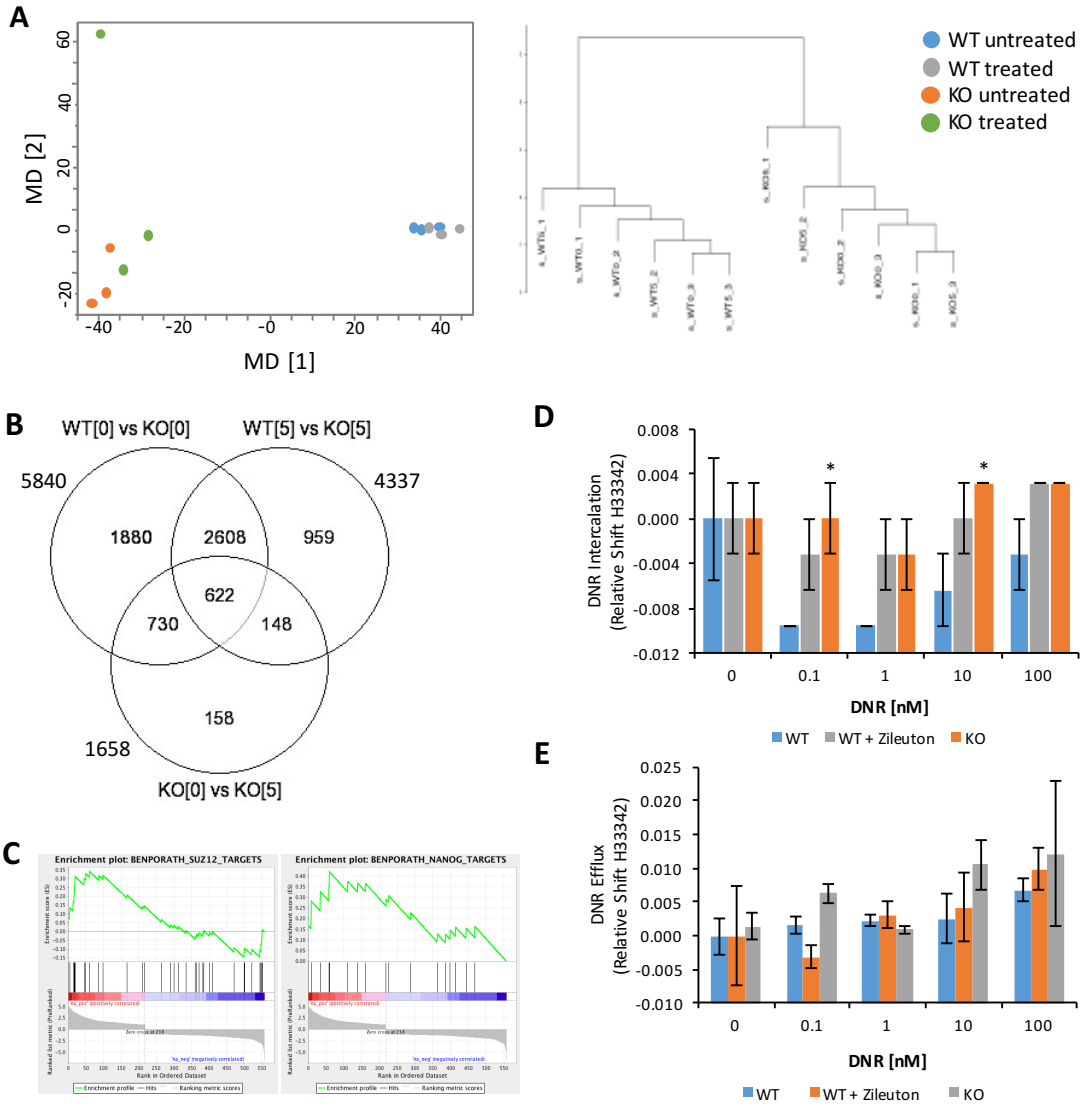
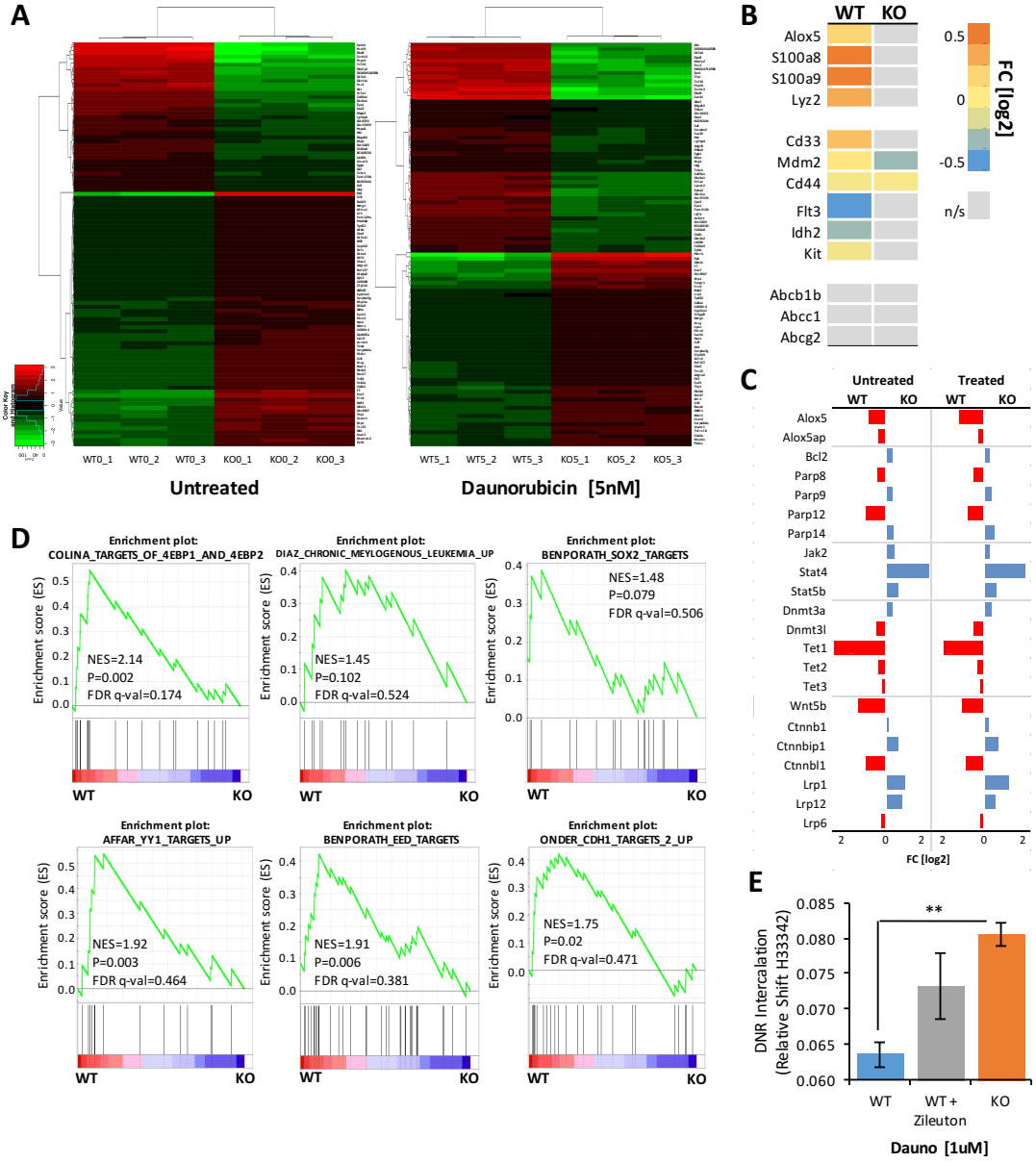

A
MD [2]
MD [1]
WT untreated
WT treated
KO untreated
KO treated
B
WT[0] vs KO[0]
WT[5] vs KO[5]
KO[0] vs KO[5]
5840
4337
1658
1880
2608
959
622
730
148
158
C
Enrichment plot: BENPORATH_SUZ12_TARGETS
Enrichment plot: BENPORATH_NANOG_TARGETS
D
DNR Intercalation
(Relative Shift H33342)
DNR [nM]
WT
WT + Zileuton
KO
E
DNR Efflux
(Relative Shift H33342)
DNR [nM]
WT
WT + Zileuton
KO

Figure 3-5: 5-LO depletion alters global transcription programs and increases DNR binding to DNA

(A) Heatmaps of differentially expressed genes between untreated WT and *Alox5*$^{-/-}$ MLL-AF9 leukemic cells (left panel) and *ex vivo* DNR treated WT and *Alox5*$^{-/-}$ MLL-AF9 leukemic cells (right panel). (B) Significantly altered (P<0.05) known and putative chemoresistance genes. (C) Baseline expression of selected genes (P-value < 0.05) in WT and *Alox5*$^{-/-}$ MLL-AF9 leukemic cells for known chemoresistance genes. (D) Enrichment plots from GSEA of pathways and targets upregulated (positive enrichment score) in WT vs *Alox5*$^{-/-}$ leukemic cells. (E) Spectral shift of Hoechst 33342 peak emission with exposure to DNR in WT and *Alox5*$^{-/-}$ MLL-AF9 leukemic cells treated *ex vivo*. The results are presented as mean ± standard error mean (SEM). P-values were determined by the Student's t-test; **P < 0.01. n/s, not significant (P>0.05).


A
WT0_1
WT0_2
WT0_3
KO0_1
KO0_2
KO0_3
WT5_1
WT5_2
WT5_3
KO5_1
KO5_2
KO5_3
Untreated
Daunorubicin [5nM]
B
WT
KO
Alox5
S100a8
S100a9
Lyz2
Cd33
Mdm2
Cd44
Flt3
Idh2
Kit
Abcb1b
Abcc1
Abcg2
0.5
0
-0.5
n/s
FC [log2]
C
Untreated
Treated
WT
KO
WT
KO
Alox5
Alox5ap
Bcl2
Parp8
Parp9
Parp12
Parp14
Jak2
Stat4
Stat5b
Dnmt3a
Dnmt3l
Tet1
Tet2
Tet3
Wnt5b
Ctnnb1
Ctnnbip1
Ctnnbl1
Lrp1
Lrp12
Lrp6
2
0
2
2
0
2
FC [log2]
D
Enrichment plot:
COLINA_TARGETS_OF_4EBP1_AND_4EBP2
NES=2.14
P=0.002
FDR q-val=0.174
Enrichment plot:
DIAZ_CHRONIC_MEYLOGENOUS_LEUKEMIA_UP
NES=1.45
P=0.102
FDR q-val=0.524
Enrichment plot:
BENPORATH_SOX2_TARGETS
NES=1.48
P=0.079
FDR q-val=0.506
Enrichment plot:
AFFAR_YY1_TARGETS_UP
NES=1.92
P=0.003
FDR q-val=0.464
Enrichment plot:
BENPORATH_EED_TARGETS
NES=1.91
P=0.006
FDR q-val=0.381
Enrichment plot:
ONDER_CDH1_TARGETS_2_UP
NES=1.75
P=0.02
FDR q-val=0.471
Enrichment score (ES)
WT
KO
E
DNR Intercalation
(Relative Shift H33342)
**
WT
WT +
Zileuton
KO
Dauno [1uM]

methylation and the DNA damage response, such as Tet1 and Parp family genes, were consistently downregulated in *Alox5*$^{-/-}$ blasts regardless of treatment status (**Figure 3-5 C**). Comparison of WT and *Alox5*$^{-/-}$ blasts also revealed increased expression of Jak2 and Stat4 in *Alox5*$^{-/-}$ cells, consistent with an inhibitory role of 5-LO in Jak/Stat signaling as previously described in other myeloproliferative neoplasms (**Figure 3-5 C**) (Y. Chen et al., 2016). GSEA profiling revealed positive enrichment of 4ebp1/2, Yy1, Eed, Sox2 and Cdh1 targets in WT leukemia, important genes in the regulation of differentiation, cell adhesion, and global transcriptional programs (**Figure 3-5 D, Figure 3-4 C**).

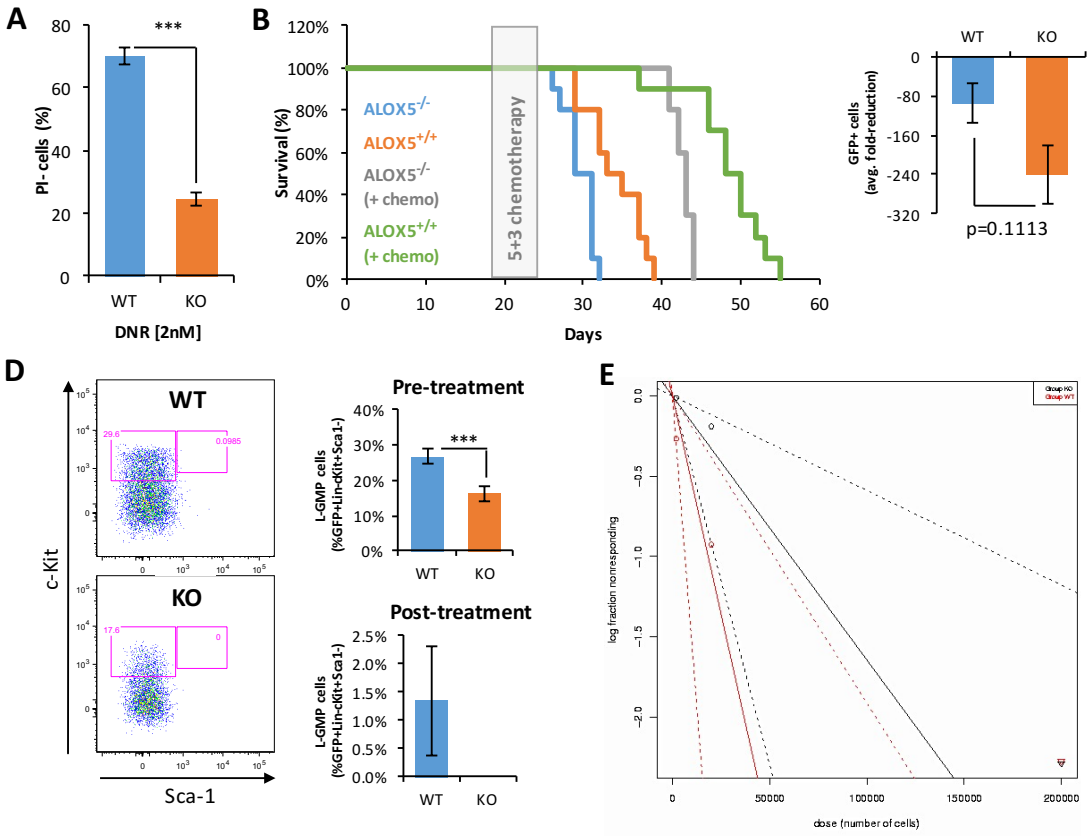
While we did not see a significant difference in ABCB1, ABCC1, or ABCG2 expression between WT and *Alox5*$^{-/-}$ blasts (**Figure 3-5 B**), given the important role of drug efflux in anthracycline resistance (Dean et al., 2005), DNR efflux and DNA intercalation were assessed. Inhibition of MLL-AF9 WT blasts with Zileuton significantly increased DNR binding to DNA to levels slightly less than in *Alox5*$^{-/-}$ blasts, a trend that was maintained over a range of DNR concentrations (**Figure 3-5 E, Figure 3-4 D**). *Alox5*$^{-/-}$ leukemic cells treated with DNR *ex vivo* exhibited increased DNA binding, but exhibited no significant difference in drug efflux (**Figure 3-4 D, E**). These data indicate that one role of leukotrienes is to limit the DNA damaging effects of DNR, primarily through preventing daunorubicin-DNA intercalation.

5-LO regulates LSC frequency and chemosensitivity *in vivo*

Consistent with our results with 5-LO knockdown (**Figure 3-1 C, H**), MLL-AF9 *Alox5*$^{-/-}$ cells treated *ex vivo* exhibited a doubling in sensitivity to DNR (**Figure 3-6 A**). To determine whether 5-LO also mediates chemoresistance *in vivo*, MLL-

Figure 3-6: 5-LO depletion sensitizes LSCs to chemotherapy in vivo

(A) PI- cells as a percent of untreated in WT and *Alox5*$^{-/-}$ MLL-AF9 leukemic cells treated with DNR *ex vivo*. (B) Kaplan-Meier curves of mouse survival harboring WT or *Alox5*$^{-/-}$ MLL-AF9 leukemia either with or without treatment for 5 days with Ara-C and 3 days with doxorubicin (n = 10). (C) Average fold-reduction in leukemic burden between *in vivo* treated WT and *Alox5*$^{-/-}$ MLL-AF9 mice (n = 4) as measured by decrease in GFP+ cell frequency. (D) Representative FACS plots of GFP+Lin-c-Kit+Sca-1- LSCs from WT and *Alox5*$^{-/-}$ MLL-AF9 mice by bone marrow aspiration (left panel), frequency of ($GFP^+Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+CD16^+$) L-GMP cells harvested before and after treatment with 5+3 induction therapy (right panel). (E) LIC frequency determination by Log fraction nonresponding as a function of number of transplanted leukemic cells in a limiting dilution assay. Mouse survival times are presented as median survival ± standard error (s.e.). *P*-values were determined by log-rank test (chi-sq). All other results are presented as mean ± standard error mean (SEM). *P*-values were determined by the Student's t-test; $^{**}P < 0.01$, $^{***}P < 0.001$.


A
PI- cells (%)
***
WT
KO
DNR [2nM]
B
Survival (%)
ALOX5-/-
ALOX5+/+
ALOX5-/- (+ chemo)
ALOX5+/+ (+ chemo)
5+3 chemotherapy
Days
GFP+ cells (avg. fold-reduction)
p=0.1113
D
WT
KO
c-Kit
Sca-1
Pre-treatment
Post-treatment
L-GMP cells (%GFP+Lin-cKit+Sca1-)
E
log fraction nonresponding
dose (number of cells)

AF9 WT and *Alox5*$^{-/-}$ AMLs were transplanted into WT mouse recipients and the resulting leukemias were treated with a modified "3+5" doxorubicin + Ara-C treatment protocol designed to mimic AML induction therapy (**Figure 3-7 A**) (Zuber et al., 2009). In agreement with our *ex vivo* analyses, *Alox5*$^{-/-}$ leukemic mice treated with chemotherapy *in vivo* exhibit a significant extension in survival compared to WT treated mice (*P*-value = 0.0022; **Figure 3-6 B**). Additionally, depletion of 5-LO alone reduces the leukemogenic potential of MLL-AF9 driven leukemia (**Figure 3-6 B**). Mice harboring comparable levels of leukemic burden (0.5% < GFP < 3%) prior to treatment display a 3-fold greater reduction in leukemic burden in *Alox5*$^{-/-}$ MLL-AF9 mice (**Figure 3-6 C, Figure 3-7 B**). Together, this suggests a role of 5-LO in mediating both leukemogenesis and resistance to induction therapy *in vivo*.

Since LSCs drive relapse in AML, we next examined whether 5-LO regulates LSC response to therapy. As functional LSCs in MLL-AF9 leukemias can be identified based on their immunophenotype, which resembles normal granulocyte-macrophage progenitors (i.e. leukemic GMP (L-GMP); Lin$^-$c-Kit$^+$Sca-1$^-$CD34$^+$CD16$^+$) (Krivtsov et al., 2006), we assessed the frequency of these cells following treatment with induction therapy *in vivo*. *Alox5*$^{-/-}$ LSCs were significantly reduced compared to WT LSCs, supporting the importance of 5-LO in their survival in the presence of chemotherapy (**Figure 3-6 D**). To further determine *Alox5*$^{-/-}$ leukemia-initiating cell (LIC) activity *in vivo*, a limiting dilution assay of *Alox5*$^{-/-}$ leukemic cells was performed in WT recipient mice. *Alox5*$^{-/-}$ blasts exhibited an LIC frequency of 1/60,780 compared to 1/18,409 in MLL-AF9 WT AML (**Figure 3-6 E**). These data are in agreement with the diminished leukemogenic capacity observed in transplanted *Alox5*$^{-/-}$ leukemia (**Figure 3-6 B, Figure 3-7 B**).

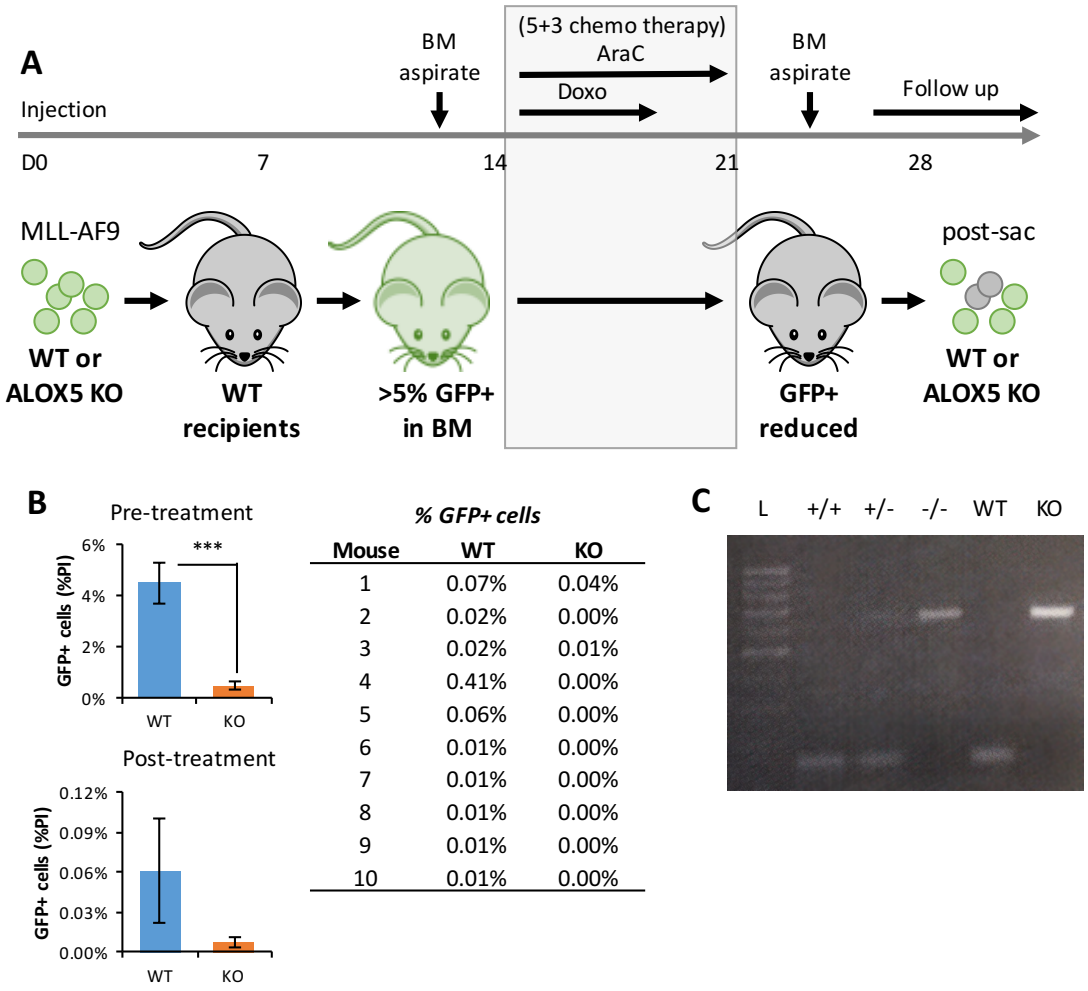


Figure 3-7: 5-LO depletion increases chemotherapeutic efficacy in vivo

(A) Workflow for establishment of MLL-AF9 leukemic mice and *in vivo* chemotherapy treatment. (B) Levels of leukemic burden as measured by GFP+ cell frequency as a percent of total PI- cells. Cells were harvested both prior to treatment (top panel) and after treatment (bottom panel) by bone marrow aspiration. (C) Genotyping of *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice to confirm presence of homozygous *Alox5* deletion. The results are presented as mean ± standard error mean (SEM). *P*-values were determined by the Student's t-test; ***$P < 0.001$.

Loss of 5-LO improves normal HSC function

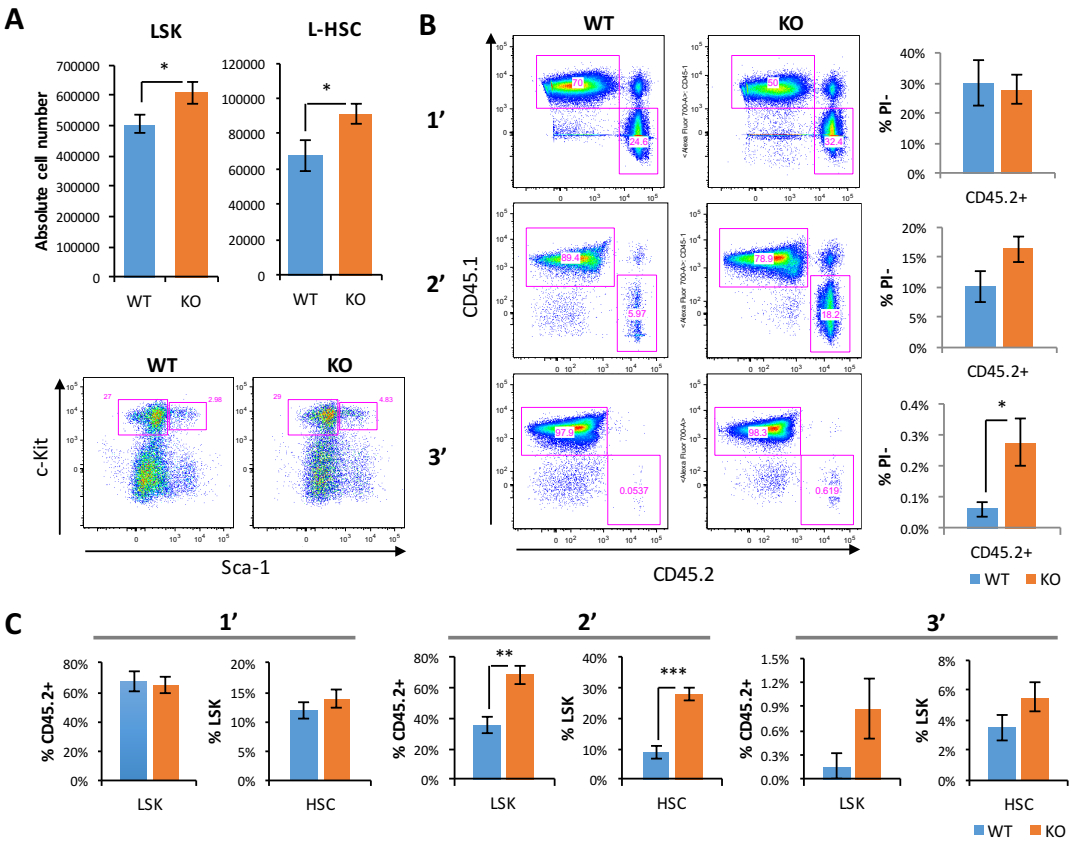
As LSC-directed therapies should exhibit specificity over HSCs, and given the defined role of leukotrienes in promoting hematopoietic progenitor proliferation and stimulation of granulocyte/macrophage production (Claesson et al., 1985; Ziboh et al., 1986), HSC maintenance in the context of 5-LO depletion was evaluated. Bone marrow from WT and *Alox5*$^{-/-}$ mice was isolated and stem cell frequency was assessed. During steady-state hematopoiesis, *Alox5*$^{-/-}$ mice exhibited higher absolute LSK (Lin$^-$c-Kit$^+$Sca-1$^+$) cells in the bone marrow, particularly the lymphoid-biased HSCs (Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$CD150$^-$) (**Figure 3-8 A**). Bone marrow cell counts and HSPC and lineage distribution were normal for all other cell types assessed (**Figure 3-9 A-C**), confirming results from a previous study (X.-S. Chen, Sheller, Johnson, & Funk, 1994). All peripheral blood counts were within the normal range (**Figure 3-10 A**), and peripheral blood lineage frequencies were comparable to WT with the exception of elevated effector T cell frequency (**Figure 3-10 B**).

Histologic sections of WT and *Alox5*$^{-/-}$ mouse BM revealed a significant increase in megakaryocytes in *Alox5*$^{-/-}$ mice (**Figure 3-11 A**). Elevated megakaryocytes were also observed in the peripheral blood of *Alox5*$^{-/-}$ mice at steady state (**Figure 3-11 B**). Despite slightly decreased total colony number, purified *Alox5*$^{-/-}$ HSCs also produced significantly more CFU-MK in primary and secondary plating than WT (**Figure 3-11 C, D**). Together these data depict grossly similar steady state hematopoiesis between 5-LO WT and KO mice, with *Alox5*$^{-/-}$ mice exhibiting subtly elevated LSK and megakaryocyte frequency.

As the increase in LSK cells in *Alox5*$^{-/-}$ mice is suggestive of a gain in HSC function, this possibility was evaluated in long-term reconstitution experiments.

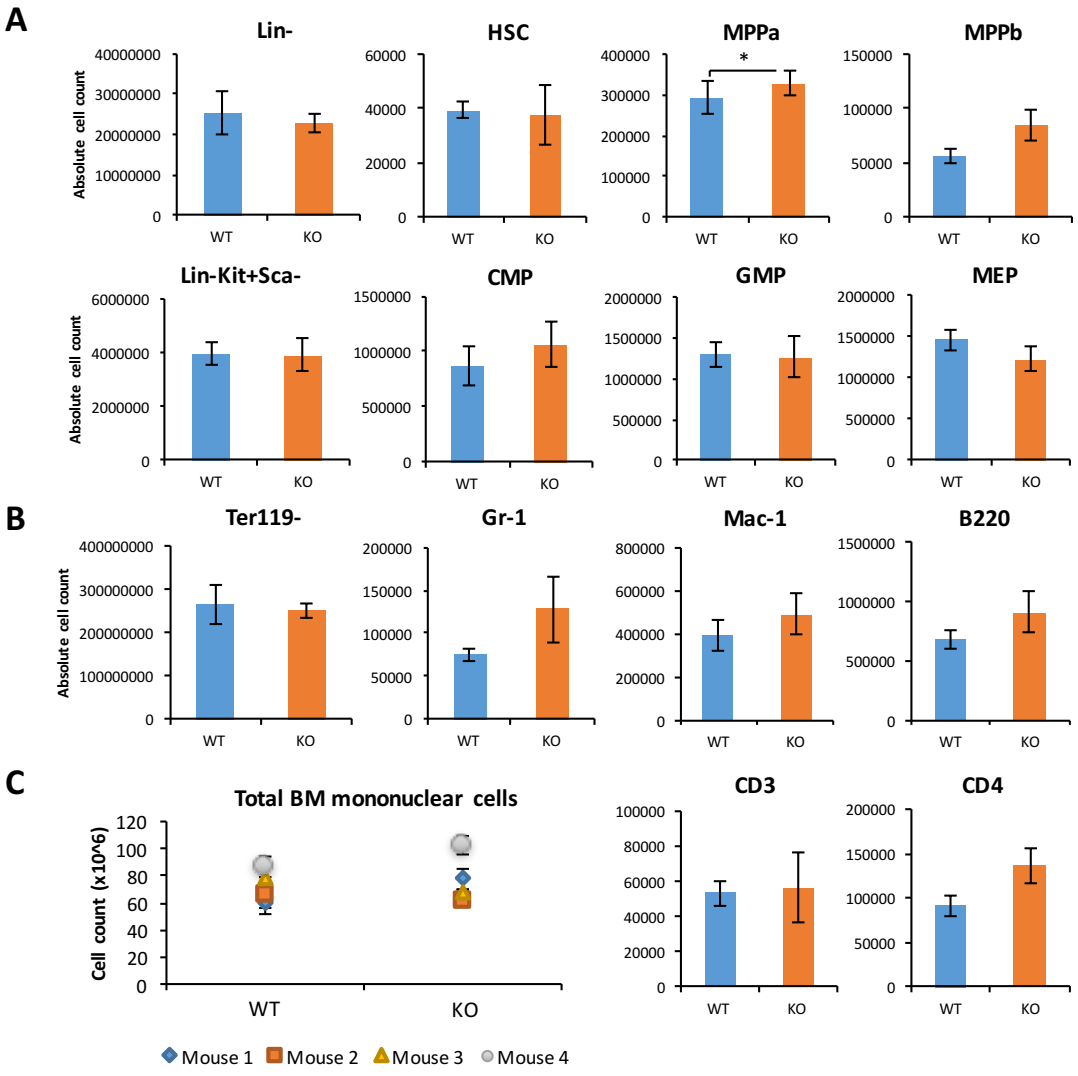
Figure 3-8: 5-LO depletion improves normal HSC function

(A) Absolute cell numbers of ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$) LSK and ($Lin^-Sca\text{-}1^+c\text{-}Kit^+CD34^-CD150^-$) L-HSC cells in the bone marrow of WT and *Alox5*$^{-/-}$ mice (top panel), representative FACS plots (bottom panel). (B) Flow cytometric scatter plot of recipient (CD45.1) and donor (CD45.2) cell chimerism in WT mice transplanted with either of WT and *Alox5*$^{-/-}$ HSCs 16 weeks after transplantation. Bar graphs depict quantitation of mean CD45.2+ donor cells as a frequency of total PI- cells. (C) LSK frequency of total CD45.2+ cells and HSC frequency of total LSK cells in mice transplanted with WT and *Alox5*$^{-/-}$ MLL-AF9 donor cells. Primary (1'), secondary (2'), and tertiary (3') transplantation. The results are presented as mean ± standard error mean (SEM). *P*-values were determined by the Student's t-test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.


A
LSK
L-HSC
Absolute cell number
WT
KO
WT
KO
WT
KO
c-Kit
Sca-1
B
WT
KO
1'
2'
3'
CD45.1
CD45.2
% PI-
CD45.2+
WT
KO
C
1'
2'
3'
% CD45.2+
% LSK
LSK
HSC
WT
KO

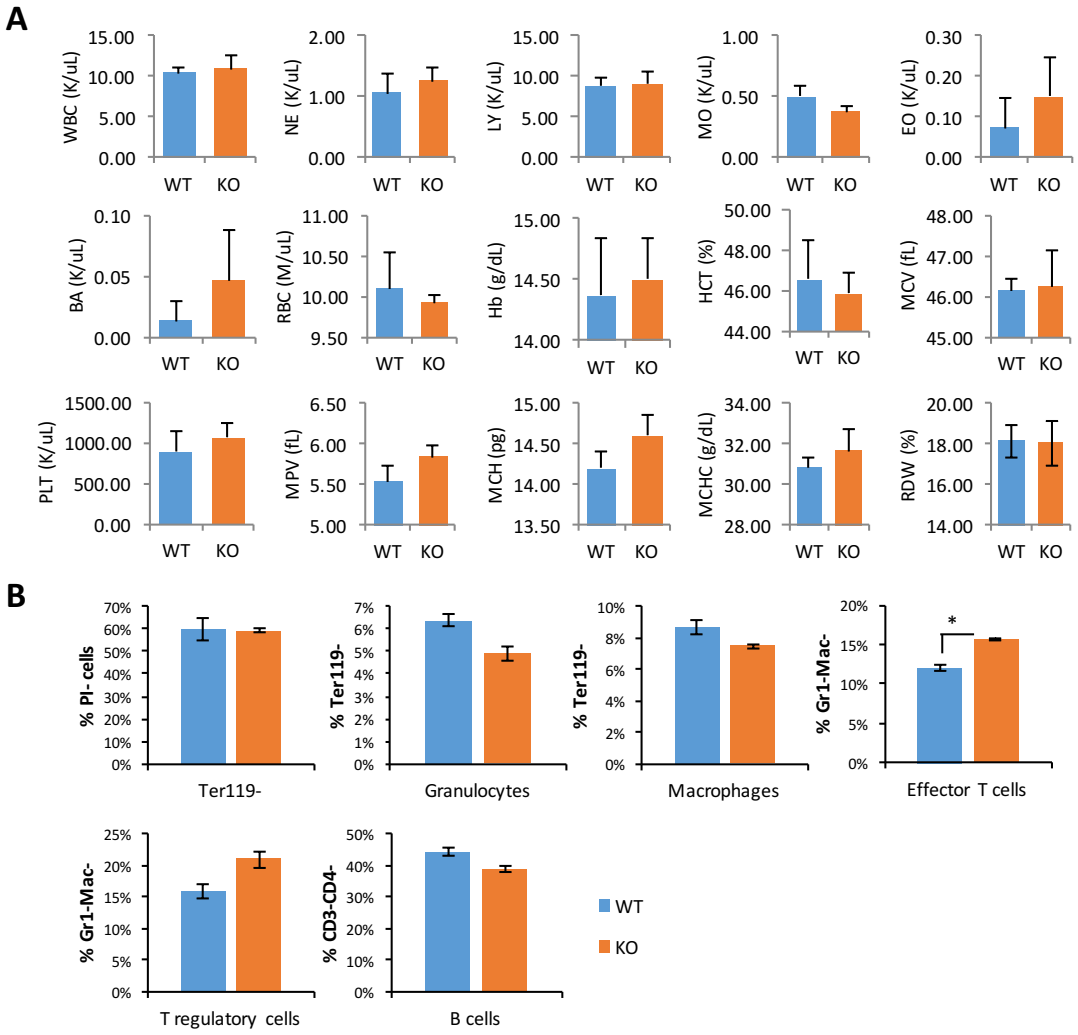
<u>Figure 3-9:</u> 5-LO KO mice have phenotypically normal bone marrow cellularity at steady-state

(A) Total cellularity of bone marrow stem and progenitor cell populations. (B) Total cellularity of differentiated myeloid and lymphoid cell populations in the bone marrow of WT and *Alox5*$^{-/-}$ mice. (C) Total bone marrow mononuclear cell counts for WT and *Alox5*$^{-/-}$ mice. The results are presented as mean ± standard error mean (SEM). *P*-values were determined by the Student's t-test; **P* < 0.05.


A
Lin-
HSC
MPPa
MPPb
Absolute cell count
WT
KO
*
Lin-Kit+Sca-
CMP
GMP
MEP
B
Ter119-
Gr-1
Mac-1
B220
C
Total BM mononuclear cells
Cell count (x10^6)
CD3
CD4
Mouse 1
Mouse 2
Mouse 3
Mouse 4

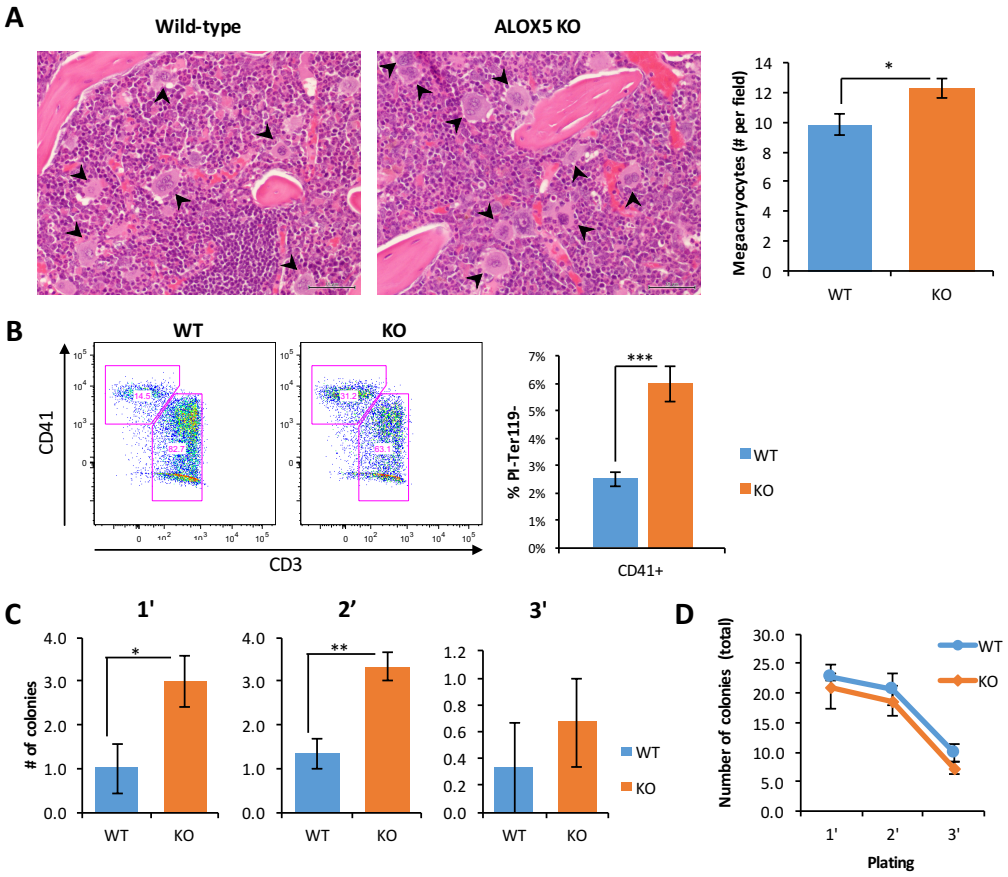
<u>Figure 3-10:</u> 5-LO KO mice have phenotypically normal peripheral blood cellularity at steady-state

(A) Complete blood counts in the peripheral blood of WT and *Alox5*$^{-/-}$ mice. (B) Frequency of differentiated myeloid and lymphoid cell populations in the peripheral blood of WT and *Alox5*$^{-/-}$ mice. The results are presented as mean ± standard error mean (SEM). *P*-values were determined by the Student's t-test; **P* < 0.05.


A
WBC (K/uL)
15.00
10.00
5.00
0.00
WT KO
NE (K/uL)
2.00
1.00
0.00
WT KO
LY (K/uL)
15.00
10.00
5.00
0.00
WT KO
MO (K/uL)
1.00
0.50
0.00
WT KO
EO (K/uL)
0.30
0.20
0.10
0.00
WT KO
BA (K/uL)
0.10
0.05
0.00
WT KO
RBC (M/uL)
11.00
10.50
10.00
9.50
WT KO
Hb (g/dL)
15.00
14.50
14.00
WT KO
HCT (%)
50.00
48.00
46.00
44.00
WT KO
MCV (fL)
48.00
47.00
46.00
45.00
WT KO
PLT (K/uL)
1500.00
1000.00
500.00
0.00
WT KO
MPV (fL)
6.50
6.00
5.50
5.00
WT KO
MCH (pg)
15.00
14.50
14.00
13.50
WT KO
MCHC (g/dL)
34.00
32.00
30.00
28.00
WT KO
RDW (%)
20.00
18.00
16.00
14.00
WT KO
B
% PI- cells
70%
60%
50%
40%
30%
20%
10%
0%
Ter119-
% Ter119-
7%
6%
5%
4%
3%
2%
1%
0%
Granulocytes
% Ter119-
10%
8%
6%
4%
2%
0%
Macrophages
% Gr1-Mac-
20%
15%
10%
5%
0%
*
Effector T cells
% Gr1-Mac-
25%
20%
15%
10%
5%
0%
T regulatory cells
% CD3-CD4-
50%
40%
30%
20%
10%
0%
B cells
WT
KO

<u>Figure 3-11:</u> 5-LO KO mice exhibit elevated megakaryocyte output in vivo and in colony formation assay

(A) Frequency of megakaryocyte frequency in the sternum of WT and *Alox5*$^{-/-}$ mice. Paraffin embedded sections were H&E stained and visualized by brightfield microscopy. Representative images shown. (B) Representative FACS plots of peripheral blood CD41+ cells from WT and *Alox5*$^{-/-}$ mice (left panel) and quantitation of average CD41+ frequency (right panel) from mice at steady state (n = 6). (C) Total colony number of purified WT and *Alox5*$^{-/-}$ HSCs plated in methylcellulose. (D) Total CFU-MK colony counts. Primary (1'), secondary (2') and tertiary (3') platings. The results are presented as mean ± standard error mean (SEM). *P*-values were determined by the Student's t-test; **P* < 0.05, ***P* < 0.01, ****P* < 0.001.


A
Wild-type
ALOX5 KO
Megacaryocytes (# per field)
WT
KO
B
WT
KO
CD41
CD3
% PI-Ter119-
CD41+
C
1'
2'
3'
# of colonies
D
Number of colonies (total)
Plating

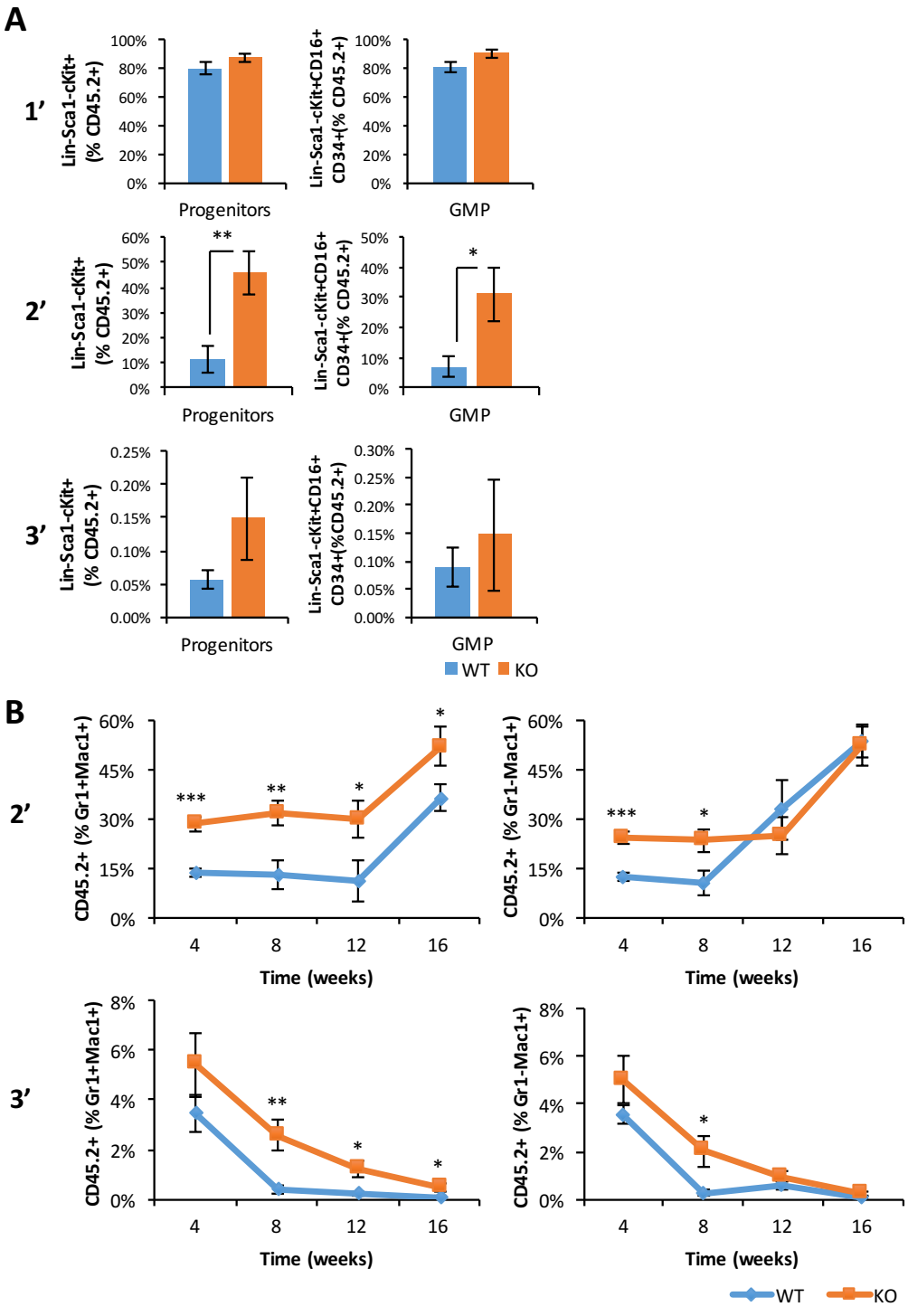
Double FACS-sorted ($Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^-CD150^+$) HSCs were transplanted into lethally irradiated WT recipients with $2.4x10^5$ cells BM MNCs as rescue cells. While no gross phenotypic abnormalities were observed in primary WT and *Alox5*$^{-/-}$ transplants, serial transplantation revealed progressive enhancement in total peripheral blood chimerism in *Alox5*$^{-/-}$ transplanted mice (**Figure 3-8 B**). Secondary *Alox5*$^{-/-}$ donor cell recipients exhibited a doubling in LSK and a tripling in HSC frequencies compared to WT (**Figure 3-8 C**). Myeloid progenitor cells ($Lin^-c\text{-}Kit^+Sca\text{-}1^-$), and particularly GMP ($Lin^-c\text{-}Kit^+Sca\text{-}1^-CD34^+CD16^+$), also exhibited elevated donor cell frequencies in secondary and tertiary recipients (**Figure 3-12 A**). The trend of increased myeloid chimerism was also evident amongst mature myeloid cells, with significantly elevated donor chimerism in peripheral blood granulocytes and macrophages in secondary and tertiary *Alox5*$^{-/-}$ recipients (**Figure 3-12 B**). Together these data suggest that 5-LO depletion improves normal HSPC function *in vivo*, with elevated myeloid cell output in serial transplantation and colony forming assays.

Alox5$^{-/-}$ HSPCs exhibit normal response to genotoxic stress

Considering the observed increase in LSC chemosensitivity, we next sought to determine whether *Alox5*$^{-/-}$ HSPCs may be more resistant to genotoxic stress. WT and *Alox5*$^{-/-}$ mice were treated with a single dose of 5-FU and the bone marrow and peripheral blood cell composition and numbers were measured weekly following treatment (Moore & Warren, 1987). The expected reduction in RBC and hematocrit was observed within seven days following treatment with 5-FU (**Figure 3-13 A**). While total lineage negative frequency increased in the bone marrow of both WT and *Alox5*$^{-/-}$ mice seven days following treatment with 5-FU, a preferential increase in

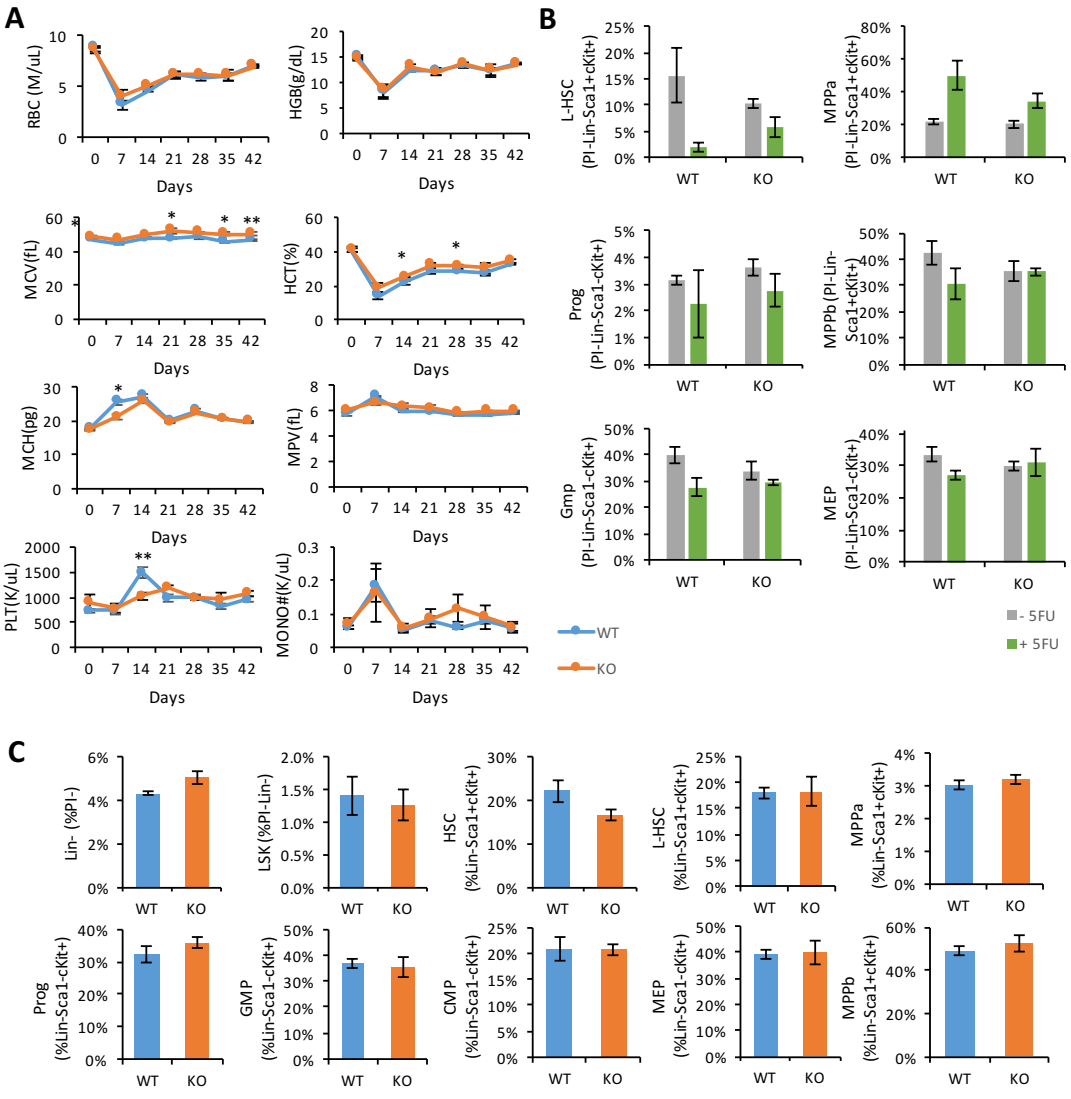
<u>Figure 3-12:</u> 5-LO KO recipients exhibit elevated progenitor and myeloid lineage output in serial transplantation

(A) Frequency of ($Lin^-Sca\text{-}1^-c\text{-}Kit^+$) progenitor and ($Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+CD16^+$) GMP cells as a percentage of total CD45.2+ WT and *Alox5*$^{-/-}$ donor cells over serial transplantations. (B) Peripheral blood CD45.2+ WT and *Alox5*$^{-/-}$ donor cell chimerism within (Gr1+Mac1+) granulocyte and (Gr1-Mac1+) macrophage cell populations. Tail bleeds were taken every 4 weeks for 16 weeks total. Primary (1'), secondary (2') and tertiary (3') transplantation. The results are presented as mean ± standard error mean (SEM) . *P*-values were determined by the Student's t-test; **P* < 0.05, ***P* < 0.01, ****P* < 0.001.


A
1'
2'
3'
Lin-Sca1-cKit+ (% CD45.2+)
Lin-Sca1-cKit+CD16+ CD34+(% CD45.2+)
Progenitors
GMP
WT
KO
B
CD45.2+ (% Gr1+Mac1+)
CD45.2+ (% Gr1-Mac1+)
Time (weeks)

<u>Figure 3-13:</u> 5-LO KO mice exhibit normal hematopoietic recovery following 5-FU treatment

(A) Total blood counts in WT and *Alox5*$^{-/-}$ mice treated with 5-FU [100mg/kg] were acquired weekly for 6 weeks. (B) Bone marrow HSPC cell frequency in WT and *Alox5*$^{-/-}$ mice 7 days (B) and 6 weeks (C) following 5-FU administration. The results are presented as mean ± standard error mean (SEM) . *P*-values were determined by the Student's t-test; **P* < 0.05, ***P* < 0.01.


A
RBC (M/uL)
HGB(g/dL)
MCV(fL)
HCT(%)
MCH(pg)
MPV(fL)
PLT(K/uL)
MONO#(K/uL)
Days
B
L-HSC (Pl-Lin-Sca1+cKit+)
MPPa (Pl-Lin-Sca1+cKit+)
Prog (Pl-Lin-Sca1-cKit+)
MPPb (Pl-Lin-Sca1+cKit+)
Gmp (Pl-Lin-Sca1-cKit+)
MEP (Pl-Lin-Sca1-cKit+)
WT
KO
- 5FU
+ 5FU
C
Lin- (%Pl-)
LSK (%Pl-Lin-)
HSC (%Lin-Sca1+cKit+)
L-HSC (%Lin-Sca1+cKit+)
MPPa (%Lin-Sca1+cKit+)
Prog (%Lin-Sca1-cKit+)
GMP (%Lin-Sca1-cKit+)
CMP (%Lin-Sca1-cKit+)
MEP (%Lin-Sca1-cKit+)
MPPb (%Lin-Sca1+cKit+)

white blood cells and leukocytes, including neutrophils, eosinophils, and basophils, was observed in the peripheral blood of WT mice compared to *Alox5*$^{-/-}$ (**Figure 3-14 A, B**). Interestingly, reticulocytes in *Alox5*$^{-/-}$ mice were relatively unaffected by 5-FU treatment, suggesting a role of 5-LO in mediating 5-FU sensitivity in this cell type (**Figure 3-14 A**). Untreated *Alox5*$^{-/-}$ mice exhibited significantly higher LSK frequency, which was reduced upon treatment with 5-FU (**Figure 3-14 B**). Following 5-FU treatment, WT and *Alox5*$^{-/-}$ 5-FU treated mice exhibited comparable bone marrow stem and progenitor cell frequencies and complete blood counts (**Figure 3-13 A-C**). While these data suggest that *Alox5*$^{-/-}$ peripheral blood and bone marrow cells are slightly more sensitive to genotoxic stress than WT, there is no long term detriment to hematopoiesis. Combined with our data in *Alox5*$^{-/-}$ leukemic mouse models, these studies suggest that 5-LO has unique functions in normal and leukemic hematopoiesis and highlight 5-LO as a tractable pharmacologic target in combination with induction therapy.

DISCUSSION

These studies identify 5-LO as a potent chemoresistance mediator in AML. While 5-LO is not expressed at significant levels prior to exposure to DNR, treatment rapidly induces expression of both 5-LO mRNA and protein, resulting in elevated leukotriene synthesis and conferral of a therapy-resistant state. This is reversed upon 5-LO knockdown or pharmacologic inhibition. Loss of 5-LO also diminished MLL-AF9-driven leukemogenesis and improved the chemosensitivity of leukemic blasts *in vivo*, with a significant survival extension observed in treated MLL-AF9 *Alox5*$^{-/-}$ mice. *Alox5*$^{-/-}$ mice exhibit elevated LSK frequency at steady state and improved HSC

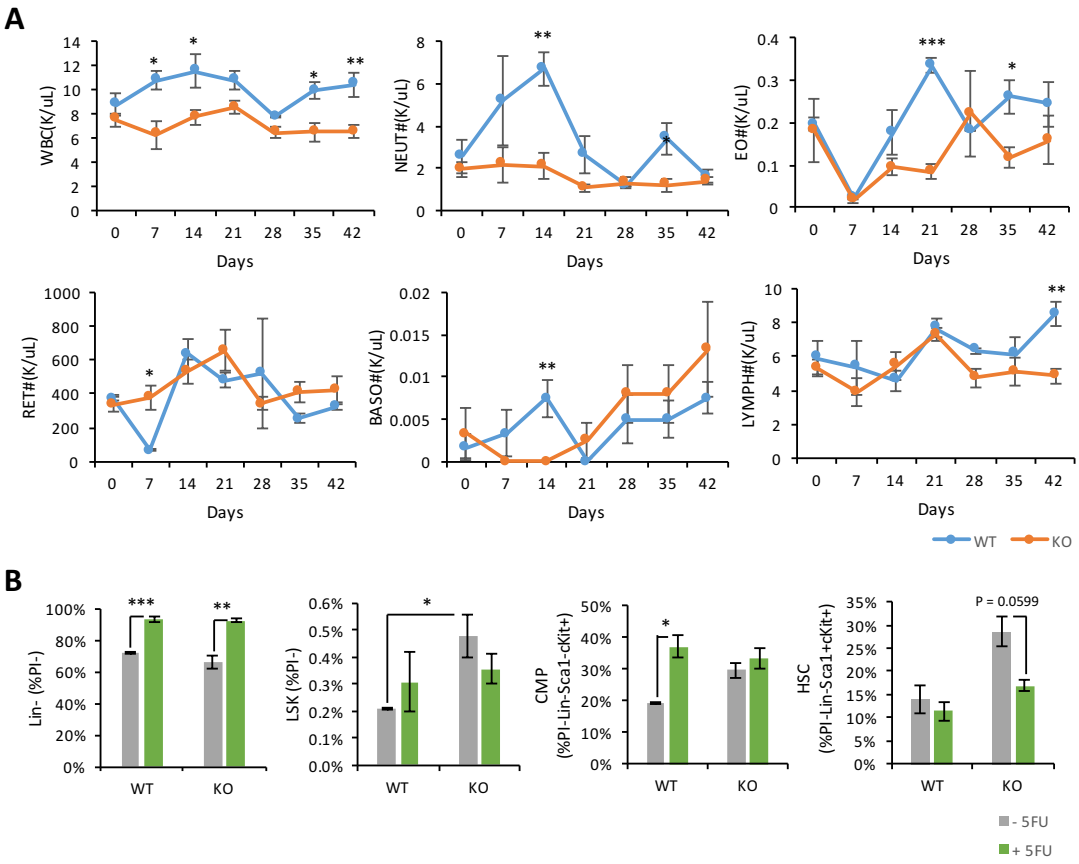


Figure 3-14: Alox5-/- mice exhibit normal hematopoietic recovery following 5-FU treatment

(A) WT and *Alox5*$^{-/-}$ mice were treated with a single dose of 5-FU and blood samples were collected by tail vein bleed weekly for 6 weeks. CBC analysis of lymphoid and leukocyte frequency are depicted. (B) Bone marrow HSPC frequencies in WT and *Alox5*$^{-/-}$ mice 6 weeks after 5-FU treatment. The results are presented as mean ± standard error mean (SEM). *P*-values were determined by the Student's t-test; **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

function in serial transplantation. Therefore, loss of 5-LO synergizes with induction therapy to improve chemoresponses, while potentially also enhancing normal hematopoietic recovery.

5-LO is a known regulator of inflammation through conversion of arachidonic acid to leukotrienes, a bioactive group of fatty acids involved in bronchoconstriction and leukocyte chemotaxis (Radmark, 2000). Altered metabolism of arachidonic acid through upregulation of 5-LO has been linked to carcinogenesis in several solid tumor models, positively regulating cancer cell viability, proliferation, cell migration, and activation of anti-apoptotic signaling cascades (Bishayee & Khuda-Bukhsh, 2013; D. Wang & Dubois, 2010). Several metabolites of arachidonic acid, including prostaglandins, are also known to protect cancer cells from different chemo-preventive measures, however the importance of leukotrienes and their relevance to AML is poorly understood (Kurtova et al., 2014; Rioux & Castonguay, 1998). Recent studies have also implicated leukotrienes in the regulation of HSPC homeostasis, though their effect on cancer stem cell growth is only beginning to be investigated (Boehmler et al., 2009; Chung et al., 2005; D. Wang & Dubois, 2010).

Our data suggest that 5-LO exerts its chemoprotective effect through the generation of leukotrienes, which in turn inhibit binding of DNR to DNA. While the inability of MLL-AF9 *Alox5*$^{-/-}$ leukemic blasts to produce leukotrienes improved their sensitivity to DNR, we also show that administration of exogenous leukotrienes can rescue the effect of 5-LO shRNA-mediated depletion. This suggests that leukotrienes may modulate chemoprotection of leukemic blasts by both cell-intrinsic and cell-extrinsic means. Further investigation of the tumor microenvironmental secretion of leukotrienes and their effect on leukemic cells is therefore required.

Prior studies in the context of a PML/RARα-positive model of AML

demonstrated that pharmacologic inhibition of 5-LO with CJ-13,610 reversed PML/RARα activation of Wnt signaling, suggesting a link between 5-LO catalytic inactivation and inhibition of Wnt as a leukotriene-independent mechanism of LSC survival (Roos et al., 2014). In the context of MLL-AF9 driven leukemia, we observe a suppression of leukotriene synthesis in Molm-13 cells induced to overexpress catalytically inactivated 5-LO, which resulted in an inability to improve chemoresistance in these cells (**Figure 3-3 C, D**). MLL-AF9 *Alox5*$^{-/-}$ leukemic blasts also did not exhibit significantly altered expression of several Wnt pathway-related genes, including: Axin1, APC, cyclin D1, and GSK3b (**data not shown**). This supports the hypothesis that 5-LO primarily mediates chemoresistance through synthesis of leukotrienes.

Curative therapies in AML must achieve complete elimination of LSCs (Horton & Huntly, 2012). Our data and data of others have shown that, while induction therapy partially reduces LSC counts, a portion of LSCs often persist, the frequency of which directly correlates with overall survival and length of remission (Stiehl et al., 2015; Terwijn et al., 2014). An attenuation in LIC frequency and an almost complete reduction in L-GMP frequency following induction therapy was observed in *Alox5*$^{-/-}$ mice *in vivo* (**Figure 3-6 D, E**). This suggests that loss of 5-LO not only decreases the number of LSCs, but also heightens the sensitivity of these remaining cells to treatment.

The combination of chemosensitization of LSCs and improved HSC function in *Alox5*$^{-/-}$ mice makes 5-LO an attractive candidate for further clinical development. Importantly, there is already an FDA-approved inhibitor of 5-LO, Zileuton, used to treat asthma (Berger, De Chandt, & Cairns, 2007). Our preliminary studies combining Zileuton with DNR showed a synergistic effect *in vitro*, likely through potentiation of

DNR binding to DNA (**Figure 3-3 E, Figure 3-5 D**). This is further supported by studies in CML that demonstrated Zileuton alone extended survival better than Imatinib in a BCR-ABL mouse model (Y. Chen, Hu, et al., 2009). As DNR results in cumulative toxicity in patients, combinatorial therapy that allows for a better therapeutic index of DNR could significantly change the treatment landscape in AML (Gianni et al., 2008). Our studies therefore offer a foundation for additional pre-clinical assessment of combining Zileuton with induction therapy for future clinical translation.

CHAPTER FOUR:
METHODS

Patients and samples

All patient studies were performed at Stanford Cancer Center (San Jose, California). AML patients were enrolled regardless of age, gender, ethnicity, AML subtype, or cytogenetics. The majority of patients received 7+3 induction therapy (Ara-C + Doxo), while a subset received alternative therapy in addition to, or as a replacement for, induction therapy. Day 0 and Day 14 blast percentages were annotated from BM analysis at Stanford's pathology laboratory. LSC (CD34+CD38-CD90-), LPC (CD34+CD38+), and blast (CD34-) cells were FACS sorted based on immunophenotype and RNA was extracted for transcriptome analysis. Summary of patient demographics and treatment response are summarized in **Table 2-1**.

Transcriptome analysis and bioinformatics

Gene expression analysis of sorted patient samples was performed on the Affymetrix U133 Plus 2.0 gene expression microarray platform (Affymetrix, Santa

Clara, California). Sample clustering analysis was performed by multidimensional scaling of all samples. Heatmaps of the top 100 differentially-expressed genes in each comparison were generated for genes meeting fold change cutoff $\log_2$, adjusted p-value cutoff 0.05, and mean coverage of at least 15. Pathway analysis was performed using the GSEA software package (Mootha et al., 2003; Subramanian et al., 2005). Differential gene expression overlap between comparisons was performed using the Gene List Venn Diagram software (Pirooznia, Nagarajan, & Deng, 2007).

In vitro cell line studies

Human cell lines (MOLM-13) were obtained from American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH (DSMZ) and were maintained according to their respective guidelines. MOLM-13 cells were lentivirally transduced with shRNAs against 5-LO depicted in **Table 4-1** in the pLKO-Puro vector backbone or vector alone as a control. Transduced clones were selected with puromycin.

Molm13 or HL-60 cells were lentivirally transduced with shRNAs against the 51 gene panel depicted in Sup. Table 2 in the pLKO-Puro vector backbone or vector alone as a control (Sup. Table 3). Transduced clones were selected with puromycin. Overexpression of *Alox5* cDNA was achieved in pLenti-Lox-GFP-mCherry vector backbone (**Table 4-1**). GFP+ transduced clones were FACS-sorted by flow cytometry and expression was induced by the addition of doxycycline and tracked by mCherry fluorescence. Catalytically inactive 5-LO was accomplished through single point mutation of HIS368SER in the cDNA of the 5-LO overexpression construct, as

Table 4-1: Primers

Target	Primer	Sequence	Remarks
ALOX5	Forward	GCAGGAAGTGGCTACTGTGGA	Common (for genotyping)
ALOX5	Forward	TGCAACCCAGTACTCATCAAG	Wild-type (for genotyping)
ALOX5	Forward	ATCGCCTTCTTGACGAGTTC	Knockout (for genotyping)
ALOX5	Forward	GTACGAATTCGTTTTCCCAGTCACGAC	Overexpression cloning
ALOX5	Reverse	GCTAACCGGTCAGGAAACAGCTATGAC	Overexpression cloning
ALOX5	Forward	GACCATCACCTCGCTTCTGCGAAC	Mutagenic PCR
ALOX5	Reverse	GTTCGCAGAAGCGAGGTGATGGTC	Mutagenic PCR
pLentiLox		GCGATACTAGAGCTTGCATGC	Sequencing primer for ALOX5 incorporation
ALOX5	Forward	ACTGGAAACACGGCAAAAAC	qPCR
ALOX5	Reverse	TTTCTCAAAGTCGGCGAAGT	qPCR
hPRT1	Forward	TCCAGCAGGTCAGCAAAGAA	qPCR
hPRT1	Reverse	GAACGTCTTGCTCGAGATGT	qPCR

previously described (**Table 4-1**) (Ishii, Noguchi, Miyano, Matsumoto, & Noma, 1992; Nguyen, Fealgueyret, Abramovitz, & Riendeau, 1991).

5-LO drug inhibition

For cytotoxicity studies, cell death was assessed after 3 days of chemotherapy treatment with DNR (Sigma, cat no: 30450). For combination with Zileuton, cells were treated with daunorubicin (7nM) for 3 days with addition of Zileuton (0-100uM, LKT Laboratories cat no: Z3444) or vehicle (DMSO) 24 hours prior to addition of daunorubicin and every 24 hours after. Cell death was assessed by addition of PI (0.5ug/mL) and flow cytometry.

Myeloablation studies were performed by intraperitoneal administration of a single 100mg/kg dose of 5-flourouracil (Sigma, cat. no: F6627-1G) to C57BL/6J WT and *Alox5*$^{-/-}$ mice. Peripheral blood samples were collected weekly for 6 weeks by tail vein bleed. Bone marrow was collected by aspiration 7 days following 5-FU administration and upon experiment termination by crushing of the femur and tibia.

Alox5$^{-/-}$ mouse studies

C57BL/6J *Alox5*$^{-/-}$ mice were obtained from The Jackson Laboratory (JAX, stock no: 004155). Homozygous deletion of *Alox5* was confirmed through genotyping of ear tissue by PCR and gel electrophoresis (**Figure 3-7 C**). Mice were maintained at Memorial Sloan-Kettering animal facility following standard protocols for animal maintenance.

Alox5$^{-/-}$ serial transplantation studies were performed by transplanting purified, double sorted (Lin^{-}c-Kit^{+}Sca-1^{+}CD34^{-}CD150^{+}) HSCs isolated from both WT and

Alox5$^{-/-}$ C57BL/6J mice. Lethally irradiated (two doses of 475cGy) WT Pepboy recipients were transplanted with 300 purified HSCs per mouse along with 2.4x10^5 Pepboy helper marrow by retro-orbital injection. Mouse blood samples were collected every 4 weeks by tail vein bleed and CBC, chimerism, and lineage output was assessed. Mice were sacrificed 16 weeks following transplantation and a full hematopoietic workup was performed, including: Bone marrow cellularity, stem/progenitor and lineage frequency, and histological staining. Secondary and tertiary transplants were performed by injected equal numbers of (Lin$^-$Sca-1$^+$c-Kit$^+$) LSK cells. Subsequent analyses were performed as in primary transplants.

Tissues from WT and *Alox5*$^{-/-}$ transplanted mice were sectioned, fixed, and paraffin embedded by the MSKCC Molecular Cytology core. H&E histological staining was performed and slides were imaged by brightfield microscopy (Zeiss Axio2Imaging microscope, 40x 0.75NA objective, Zeiss AxioCam HRc camera). Complete blood counts were acquired using the IDEXX Procyte Dx® Hematology Analyzer (IDEXX Laboratories)

Colony formation assays were performed by initial plating of 70 purified, double sorted (Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$CD150$^+$) HSCs per well in triplicate. Colonies were allowed to form for 7-10 days and CFUs were manually scored. Secondary and tertiary platings were performed with 2x10^4 cells homogenized from prior platings.

Alox5$^{-/-}$ MLL-AF9 studies

Leukemic mice were generated through retroviral incorporation of the MLL-AF9 oncogene fusion as previous published(Krivtsov et al., 2006). Double sorted Lin$^-$ Sca-1$^+$c-Kit$^+$ cells (LSK) isolated from the bone marrow of either C57BL/6J *Alox5*$^{+/+}$

or *Alox5*$^{-/-}$ mice were transduced and sorted GFP+ cells were transplanted into primary C57BL/6J recipients. Upon leukemic expansion, leukemic mice were sacrificed, bone marrow was isolated, and cells were taken for secondary transplantation into C57BL/6J recipients. Leukemogenesis studies and *in vivo* chemotherapy treatment were performed with tertiary transplant recipients.

MLL-AF9 *Alox5* WT/KO mouse leukemic bone marrow cells were cultured *ex vivo* in RPMI media supplemented with 10% FBS, 1% penicillin/streptomycin/L-glutamine (Gibco, cat no: 10378016), mIL-3 (10ng/mL), mSCF (20ng/mL), and mIL-6 (10ng/mL). Fresh cells were plated from frozen aliquots after 15-20 passages.

A modified induction therapy regimen developed for mice containing Ara-C and Doxorubicin (doxo) "5+3" chemotherapy was used for *in vivo* chemoresistance studies as previous described (Zuber et al., 2009). WT C57BL/6J mice at 10-12 weeks of age (The Jackson Laboratory) were sub-lethally irradiated at 475cGy and transplanted with either MLL-AF9 *Alox5*$^{+/+}$ or MLL-AF9 *Alox5*$^{-/-}$ cells ($6x10^5$ GFP+ cells) 25 hours after irradiation by retro-orbital injection. GFP+ engraftment was assessed by bone marrow aspiration 16 days post-transplantation. Chemotherapeutic treatment was initiated when 3-5% GFP+ was detected in the bone marrow. Treatment consisted of a combination of cytarabine (100mg/kg, LKT Laboratories cat no: C9778) and doxorubicin (3mg/kg, LKT Laboratories cat no: D5794). Drugs were administered by intraperitoneal injection every 24 hours for 5 days or 3 days for cytarabine and doxorubicin, respectively. Reduction in GFP+ leukemic burden was assessed 3 days after conclusion of treatment by bone marrow aspiration and flow cytometry. End point measurement included death of mice from leukemia or resulting from a humane endpoint when mice suffered from paralysis, severe stress, or cachexia.

Limiting dilution studies were performed by retro-orbital injection of $\log_{10}$

increasing concentrations from $2x10^3$ to $2x10^5$ of WT MLL-AF9 and *Alox5*$^{-/-}$ MLL-AF9 GFP+ leukemic bone marrow cells into sublethally irradiated (475cGy) C57BL/6 mice. Leukemia-initiating cell frequencies were ascertained using the Extreme Limiting Dilution Analysis (ELDA) software (Hu & Smyth, 2009).

Flow cytometry

Cell sorting and analysis was performed using the BD FACSAria II sorter (BD Biosciences) or BD LSRFortessa (BD Biosciences), respectively. Generally, antibody staining was performed for 60 minutes at 4C in the dark. For cytotoxicity studies, propidium iodide (PI) and a fixed number of unstained beads were added and a ratio of live cell and bead counts were normalized to untreated controls (**Table 4-2**). Measurement of cell cycle status was performed using anti-Ki67 Kit (BD Pharmingen, cat no: 556027). Cells were fixed and permeabolized using BD Cytofix/Cytoperm (BD Pharmingen, cat no: 554714) by manufacturer's specifications. Antibodies and other staining molecules used in all studies are depicted in **Table 4-3**. Flow cytometry data analysis was performed using FlowJo 9.8.5 software (FlowJo, LLC).

Daunorubicin intercalation and efflux were measured as previously described (Belloc et al., 1992; Smeets et al., 1999). Briefly, spectral shift of Hoechst 33342 (5uM) upon daunorubicin (0-100nM) intercalation to DNA in cell lines *in vitro* was measured using a 450 band pass (BP) filter and a 575 BP filter, respectively. Transmission efficiency was calculated to quantitate relative amount of daunorubicin bound. Daunorubicin efflux was calculated by performing a pulse-chase of daunorubicin (0-1uM) for 60 minutes at 37C, washing cells, and then incubating with H33342 for 120 minutes at 37C, followed by flow cytometric analysis.

Table 4-2: DNA dyes

Dye	Concentration	Use	Source	Catalog No.
DAPI	0.01µg/mL	Cytotoxicity assay	Fisher Scientific	26-829-810MG
PI	0.5µg/mL	Cytotoxicity assay	Sigma-Aldrich	81845
H33342	5µM	Daunorubicin DNA binding assay	Sigma-Aldrich	B2261-100MG

Table 4-3: Antibodies

Antigen	Conjugate	Clone	Use	Source	Catalog No.
c-Kit	PE/APC	2B8	Flow Cytometry	Biolegend	105808/ 105812
Sca1	PerCP	D7	Flow Cytometry	Biolegend	108122
CD34	APC	581	Flow Cytometry	Biolegend	343510
CD38	PE	DL-101	Flow Cytometry	Biolegend	352306
CD16/32	A700	93	Flow Cytometry	eBioscience	56-0161-82
Mac-1	PB	M1/70	Flow Cytometry	eBioscience	48-0112-82
Gr-1	PE-Cy7/PE	RB6-8C5	Flow Cytometry	Biolegend/ eBioscience	108416/ 12-5931-82
B220	PE-Cy5	RA3-6B2	Flow Cytometry	Biolegend	103210
CD3	APC-Cy7	17A2	Flow Cytometry	eBioscience	47-0032-82
Ter119	PE-Cy7	TER-119	Flow Cytometry	Biolegend	116222
Ki67	PE	B56	Flow Cytometry	BD Biosciences	556027
ALOX5	-	Rabbit monoclonal	Primary in Western Blot	Cell Signaling	3289S
β-actin	-	Rabbit monoclonal	Primary in Western Blot	Cell Signaling	4970S
Rabbit IgG	HRP	Polyclonal	Secondary in Western Blot	Cell Signaling	7074P2

RNA analysis

Changes in RNA transcript (CST7, CD36, 5-LO, S100A8, S100A9, LYZ) were quantitated by qPCR. RNA was extracted from cells (Qiagen RNeasy Mini Kit, cat no: 74104) and cDNA was generated (ThermoFisher SuperScript III, cat no: 18080051) by manufacture's specifications. qPCR was performed (ThermoScientific ABSolute Blue SYBR Green ROX Mix, cat no: AB-4162/B) with primers specific to the transcript of interest (**Table 4-1**). The $\Delta\Delta C_T$ method was used to calculate differences in gene expression. Briefly, C_T was measured by qPCR and expression of the gene of interest was normalized to hPRT1. Normalized values were then weighted to a reference sample and expression fold change was determined by converting from $\log_2$. Each reaction was performed in triplicate.

For RNA-sequencing analysis, WT MLL-AF9 and *Alox5*$^{-/-}$ MLL-AF9 leukemic cells were treated *ex vivo* with 5nM DNR for 72 hrs. RNA was extracted from cells (Qiagen RNeasy Mini Kit, cat no: 74104) and paired-end RNA-seq was performed by Illumina® Hiseq™ with SMARTer amplification and a read depth of 30-40x10^6 reads per sample. Alignment metrics for each sample were calculated by GATK's CollectRnaSeqMetrics and AlignmentSummaryMetrics. Sample clustering analysis was performed using either hierarchical clustering or multidimensional scaling of all samples. Heatmaps of the top 100 differentially-expressed genes in each comparison were generated for genes meeting fold change cutoff $\log_2$, adjusted p-value cutoff of 0.05, and mean coverage of at least 15. Pathway analysis was performed using the GSEA software package (Mootha et al., 2003; Subramanian et al., 2005). Differential gene expression overlap between comparisons was performed using the Gene List Venn Diagram software (Pirooznia et al., 2007).

Protein analysis

Cells were centrifuged at 1500rpm for 5 minutes at 4C (Eppendorf Centrifuge 5810 R). Cell pellets were resuspended in 100ul NP-40 lysis buffer (10mM HEPES pH 7.9, 250mM NaCl, 5mM EDTA, 0.1% NP-40, 10% Glycerol) and incubated on ice for 30 minutes. Lysates were spun at 13000rpm for 10 minutes at 4C and supernatants were extracted. Protein concentrations were quantitated by Bradford assay with BSA standard curves. Proteins were denatured in LDS buffer (C.B.S. Scientific ClearPage, cat no: FB31010) at 95C for 5 minutes and loaded onto SDS-PAGE pre-cast gels (4-20% Mini-PROTEAN TGX Precast Protein Gel, cat no: 4561094). Blots were transferred to 0.45uM nitrocellulose membrane, blocked with non-fat milk (BioRad) or 5% bovine serum albumin (Sigma), and washed with 0.1% Tween 20 tris-buffered saline. Primary and secondary antibodies used are listed in **Table 4-3**. ECL Western blotting detection reagents (Millipore) were used.

LTB4 and cysteinyl leukotriene synthesis was measured by Enzo LTB4 ELISA kit (Enzo Life Sciences, cat no: ADI-900-068) and Enzo Cysteinyl leukotriene ELISA kit (Enzo Life Sciences, cat no: ADI-900-070), respectively. Cells were stimulated 30 minutes prior to extraction of supernatant with calcium ionophore (320nM, Sigma cat no: C7522) and arachidonic acid (16uM, Sigma cat no: 10931). ELISA was performed by manufacturer's specifications. Leukotriene add-back experiments were performed as follows: MOLM-13 cells were incubated in the presence of daunorubicin (5nM) for 3 days with addition of LTB4 (4nM, Caymen Chemical cat no: 20110), LTC4 (100nM, Caymen Chemical cat no: 20210), LTD4 (100nM, Caymen Chemical cat no: 20310), and LTE4 (100nM, Caymen Chemical cat no: 20410) every 24 hours. Live (PI-) cells were quantified by flow cytometry.

CHAPTER FIVE:
DISCUSSION

Acute myeloid leukemia (AML) is a disease characterized by an accumulation of genetic aberrations that lead to a clonal outgrowth of incompletely differentiated myeloid blast cells, resulting in a progressive loss of normal hematopoietic ability. While the exact cell of origin for AML is still disputed (Pollyea et al., 2014), it is clear that the leukemic stem cell (LSC) has the ability to initiate both primary and relapsed leukemia (Somervaille & Cleary, 2006; Van Rhenen et al., 2007). Improved methods of LSC identification and isolation have allowed for better characterization of this relatively chemoresistant population (Blair et al., 1997; Bonnet & Dick, 1997; Jordan et al., 2000). In this thesis we demonstrate the first example of prospective isolation and transcriptomal analysis of paired diagnosis:post-treatment LSC populations from AML patients. Furthermore, our studies identified and experimentally validated multiple novel chemoresistance mediators *in vitro* and *in vivo*. Our approach not only offers a platform for characterization of additional LSC chemoresistance mediators in AML, but also a methodology that can be applied to the study of therapy resistance in other neoplasms.

This work addressed several previously unanswered questions in the field: 1)

How do LSCs change in response to chemotherapy *in vivo*? 2) Do LSCs primarily rely on canonical chemoresistance pathways such as drug efflux, quiescence, and anti-apoptosis? 3) Is baseline gene expression or induced gene expression more telling of ability to evade chemotherapy? Additionally, as it relates to the biology of 5-LO: 4) Does 5-LO improve leukemic cell survival through leukotrienes or another acquired process? 5) How does genetic loss of 5-LO impact normal HSC function *in vivo*? Insights to these questions obtained from our investigations are expanding upon below.

Our data demonstrate that expression of several canonical chemoresistance genes such as ABC-family transporters and cell death antagonists Bcl-2, Bcl-xL, and Mcl-1 do not significantly change in LSCs following induction therapy, despite consistently high levels at baseline. Indeed, several ABC-family transporters including: ABCC1, ABCB7, and ABCD4 were in fact downregulated in patient LSCs following induction therapy. While this does not necessarily invalidate these genes as chemoresistance mediators in AML, we identify a subset of previously unrecognized genes, including 5-LO, S100A8/9, and CD36 that are upregulated in LSCs in response to chemotherapy. We see that leukemic cell lines which can more efficiently induce expression of 5-LO are more resistant to chemotherapy *in vitro*. Many of these cell lines also demonstrate relatively low levels of 5-LO expression at baseline. This is one potential reason why many of the genes identified by our studies do not exhibit prognostic significance based on expression at diagnosis in ECOG and TCGA (Slovak et al., 2015; Voigt & Reinberg, 2013a). Therefore, our data suggest that confining prognostication of patients based solely on gene expression at diagnosis may not be the best method of predicting response to therapy. Work is currently underway in our laboratory to assess the ability of our induced LSC gene expression signature to

predict CR and length of remission compared with other published LSC and bulk leukemia gene signatures (Gentles et al., 2012; Ng et al., 2016).

We see that rather than withdrawing from the cell cycle, which is thought to be one method of LSC evasion of chemotherapy (Guzman et al., 2001; Pietras et al., 2011), LSC gene expression reflects a more proliferative state immediately following induction therapy. This is the first example indicating that LSCs are primed to expand immediately following chemotherapy in patients. This underscores the importance of post-induction consolidation and suggests that the process of relapsed leukemic outgrowth may begin as soon as therapeutic pressure is removed.

Additionally, we show through mutagenesis of the catalytic residues within the active site of 5-LO that the leukotriene-synthetic function of 5-LO is required for conferral of chemoresistance. Although prior data regarding the role of leukotrienes in leukemia are controversial (Y. Chen, Hu, et al., 2009; Roos et al., 2014), our data suggests that they are important primary mediators of chemoresistance in AML. Our approach offers several distinct advantages: Whereas previous publications used constitutive 5-LO overexpression, small molecule-mediated catalytic inactivation of 5-LO, or assessment of 5-LO inhibition in LSCs prior to transplantation, we generated ALOX5$^{-/-}$ leukemic mice and directly assessed changes in LSC chemoresponses *in vivo*. We catalytically inactivated 5-LO through mutagenic PCR, which eliminated the possibly of non-specific interactions inherent with use of small molecules. Importantly, we correlated all of our studies with changes in leukotriene synthesis and directly tested the effect of exogenous leukotriene administration on resistance to DNR. Together, our data demonstrate the sufficiency of genetic deletion of 5-LO in improving LSC chemosensitivity *in vivo* and highlight the role of leukotrienes in chemoresistance by multiple approaches.

Our studies also expand upon the only partially characterized role of 5-LO in the hematopoietic system. While our data are in agreement with published reports showing relative normalcy of ALOX5$^{-/-}$ mouse total blood cell populations and bone marrow cellularity at steady state (X.-S. Chen et al., 1994), we see a marked improvement in ALOX5$^{-/-}$ HSC function in long-term reconstitution and maintained hematopoietic recovery during 5-FU treatment. Our data therefore suggest that therapeutic targeting of 5-LO in combination with genotoxic therapies such as induction therapy is not likely to confer increased normal cell toxicity.

Together, our data identify several novel chemoresistance genes in AML with known involvement in a wide variety of biologic processes, from inflammation to lipid metabolism to protein turnover. These genes are also upregulated in a breadth of diseases, including: Diabetes, infection, asthma, and many solid tumors. The question is therefore: How can genes involved in such disparate biological processes share commonality in LSC response to chemotherapy? One potential unifying link is that these genes also have demonstrated importance in the hematopoietic system, including their pattern of cellular expression, role in hematopoiesis, and regulation of HSC maintenance. Our studies in the context of AML, a disease driven by LSCs resulting in incomplete myeloid differentiation, are therefore not completely unexpected. Based on the known positive regulation of these genes in disease pathogenesis and dependence within the hematopoietic system, we postulate that the LSC chemo-responsive gene expression signature identified by our studies is a reflection of aberrant induction of non-canonical, hematopoietic-specific cell survival pathways. Therefore, therapies designed for the elimination of LSCs should acknowledge the unique nature of the cells, environment, and functions of the hematopoietic system.

In this context, a “one size fits all” approach through targeting of drug efflux

pumps or global cell death pathways, may not yield the desired clinical outcomes, as exemplified by the inability of MDR inhibitors to improve chemoresponses in patients. Indeed, gene expression profiling of 380 known chemoresistance genes in a cohort of 11 relapsed AML patients showed significant inter-patient variably in their mechanism of chemoresistance (C. Patel et al., 2013). This highlights that a tailored assessment of a patient's particular chemoresistance profile, in addition to consideration of LSC-specific chemoresistance pathways, may be required to improve treatment. Undoubtedly, through increased understanding of the process by which LSCs originate and drive leukemia, additional therapeutic targets in AML will be identified.

While our data gives an unprecedented glimpse into the gene expression characteristics of LSC, LPC, and blast cells immediately following induction therapy in AML patients, it has also presented several additional questions that require further investigation. The question of whether cell-intrinsic or cell-extrinsic chemoresistance mechanisms have a greater influence on LSC chemotherapy evasion has so far remained unanswered. In our validation studies we see that *in vitro* treatment with cytarabine and daunorubicin directly upregulates putative chemoresistance genes in leukemic cells. This suggests that, at least for a subset of genes, leukemic cells respond in a cell-intrinsic manner to chemotherapy. Conversely, we observe an ability of exogenously administered leukotrienes to rescue the chemosensitization of 5-LO knockdown. These cells were also unable to induce 5-LO to levels comparable with control cells upon treatment with DNR. This highlights that, while the ability to induce expression of chemoresistance mediators is an important means of innate leukemic cell survival, presence of cell-extrinsic chemoresistance molecules may also be sufficient to evade therapy. Further investigation of the role of the

microenvironment in protecting LSCs from chemotherapy, particularly in relation to leukotrienes secreted by leukocytes and BM epithelia, is therefore required.

We have also shown that combination of zileuton with DNR improves human AML cell line elimination *in vitro*. This data is promising as zileuton is already FDA-approved for the treatment of asthma, with extensive safety and efficacy studies having previously been performed in humans (Berger et al., 2007; Carter et al., 1991). This makes the path for clinical translation of 5-LO inhibition in combination with induction therapy significantly more straightforward. We have therefore initiated additional pre-clinical studies in leukemia-bearing mice for the assessment of zileuton to improve chemo-responses *in vivo*, the results of which have the potential to fuel clinical trials in AML patients.

While modulation of individual chemoresistance genes in our study yielded marked increases in sensitivity to chemotherapy, our approach was limited by investigation of these genes in isolation. For future clinical development, it is possible that parallel inhibition of several chemoresistance mechanisms will be required to significantly improve patient responses to induction therapy. Experiments should therefore be performed combining inhibition of multiple candidate genes *in vitro* and *in vivo*. Indeed, a hint of the potential interdependence between the genes identified by our studies came from gene expression analysis of MLL-AF9 WT and *Alox5*$^{-/-}$ leukemic cells. While we see an induction of 5-LO, S100A8, S100A9, and Lyz2 in MLL-AF9 WT cells upon treatment with DNR, expression of these genes was not significantly altered in *Alox5*$^{-/-}$ blasts. Several other known LSC chemoresistance mediators such as CD33, Flt3, and c-Kit were also not significantly changed in treated *Alox5*$^{-/-}$ cells. This suggests that signaling downstream of 5-LO may proliferate signaling in additional chemoresistance pathways.

In sum, our studies offer an unparalleled perspective into LSC biology in response to chemotherapy. Our transcriptome analysis of patient LSCs not only provides a glimpse into how LSCs change following therapy, but has also identified and validated several novel chemoresistance genes with known inhibitors already in development. We show that inhibition of one chemoresistance mediator, 5-LO, improves chemo-responses *in vitro* and *in vivo,* while potentially preserving normal HSPC function. Therefore, our work adds further depth to our understanding of LSCs and provides actionable targets for improvement of induction therapy. Looking forward, increased understanding of how the chemoresistance pathways identified by our studies intersect in patients will allow for rational design of additional combinatorial therapies for the treatment of AML, with the ultimate goal being improved treatment responses and durations of remission.

REFERENCES

Abdel-wahab, O., & Levine, R. L. (2013). Mutations in epigenetic modifiers in the pathogenesis and therapy of acute myeloid leukemia. *Epigenetics in Hematology*, *121*(18), 3563–3572. https://doi.org/10.1182/blood-2013-01-451781.

Al-Mawali, A. (2013). Leukemic Stem Cells Shows the Way for Novel Target of Acute Myeloid Leukemia Therapy. *Journal of Stem Cell Research & Therapy*, *3*. https://doi.org/10.4172/2157-7633.1000151

Alcalay, M., Orleth, A., Sebastiani, C., Meani, N., Chiaradonna, F., Casciari, C., … Pelicci, P. G. (2001). Common themes in the pathogenesis of acute myeloid leukemia. *Oncogene*, *20*(40), 5680–5694. https://doi.org/10.1038/sj.onc.1204642

Angstreich, G. R., Matsui, W., Huff, C. A., Vala, M. S., Barber, J., Hawkins, A. L., … Jones, R. J. (2005). Effects of imatinib and interferon on primitive chronic myeloid leukaemia progenitors. *British Journal of Haematology*, *130*(3), 373–381. https://doi.org/10.1111/j.1365-2141.2005.05606.x

Antonchuk, J., Sauvageau, G., & Humphries, R. K. (2002). HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell*, *109*(1), 39–45. https://doi.org/10.1016/S0092-8674(02)00697-9

Appelbaum, F. R., Gundacker, H., Head, D. R., Slovak, M. L., Willman, C. L., Godwin, J. E., … Petersdorf, S. H. (2015). Age and acute myeloid leukemia. *Blood*, *107*(9), 3481–3486. https://doi.org/10.1182/blood-2005-09-3724.Supported

Arber, D. A., Orazi, A., Hasserjian, R., Borowitz, M. J., Beau, M. M. Le, Bloomfield, C. D., … Vardiman, J. W. (2016). The 2016 revision to the World Health Organization classi fi cation of myeloid neoplasms and acute leukemia. *Blood*, *127*(20), 2391–2406. https://doi.org/10.1182/blood-2016-03-643544.The

Balkwill, F., Charles, K. A., & Mantovani, A. (2005). Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell*, *7*(3), 211–217. https://doi.org/10.1016/j.ccr.2005.02.013

Baniwal, S. K., Khalid, O., Gabet, Y., Shah, R. R., Purcell, D. J., Mav, D., … Frenkel, B. (2010). Runx2 transcriptome of prostate cancer cells: insights into invasiveness and bone metastasis. *Molecular Cancer*, *9*, 258. https://doi.org/10.1186/1476-4598-9-258

Belloc, F., Lacombe, F., Dumain, P., Lopez, F., Bernard, P., Boisseau, M. R., & Reifers, J. (1992). Intercalation of Anthracyclines Into Living Cell DNA Analyzed by Flow Cytometry. *Cytometry*, *13*, 880–885.

Bendall, L. J., Bradstock, K. F., & Gottlieb, D. J. (2000). Expression of CD44 variant exons in acute myeloid leukemia is more common and more complex than that observed in normal blood, bone marrow or CD34 cells. *Leukemia*, *14*(7), 1239–1246. https://doi.org/10.1038/sj.leu.2401830

Benito, J., Zeng, Z., Konopleva, M., & Wilson, W. R. (2013). Targeting hypoxia in the leukemia microenvironment. *International Journal of Hematologic Oncology*, *2*(4), 279–288. https://doi.org/10.2217/IJH.13.32

Bennett, C. F., Chiang, M., Monia, B. P., & Crooke, S. T. (1993). Regulation of 5-lipoxygenase and 5-lipoxygenase-activating protein expression in HL-60 cells. *Biochem. J.*, *39*, 33–39.

Berger, W., De Chandt, M. T. M., & Cairns, C. B. (2007). Zileuton: clinical implications of 5-Lipoxygenase inhibition in severe airway disease. *International Journal of Clinical Practice*, *61*(4), 663–76. https://doi.org/10.1111/j.1742-1241.2007.01320.x

Bishayee, K., & Khuda-Bukhsh, A. (2013). 5-Lipoxygenase Antagonist therapy: a new approach towards targeted cancer chemotherapy. *Acta Biochimica et ...*, *45*, 709–719. https://doi.org/10.1093/abbs/gmt064.Advance

Blair, a, Hogge, D. E., Ailles, L. E., Lansdorp, P. M., & Sutherland, H. J. (1997). Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo. *Blood*, *89*(9), 3104–3112. https://doi.org/10.1056/nejm198708203170802

Blank, U., Karlsson, G., & Karlsson, S. (2008). Signaling pathways governing stem-cell fate. *Blood*, *111*(2), 492–503. https://doi.org/10.1182/blood-2007-07-075168

Boehmler, A. M., Drost, A., Jaggy, L., Seitz, G., Wiesner, T., Denzlinger, C., … Möhle, R. (2009). The CysLT1 ligand leukotriene D4 supports alpha4beta1- and alpha5beta1-mediated adhesion and proliferation of CD34+ hematopoietic progenitor cells. *Journal of Immunology (Baltimore, Md. : 1950)*, *182*(11), 6789–98. https://doi.org/10.4049/jimmunol.0801525

Bonnet, D., & Dick, J. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine*, *3*(7), 730–737.

Bourbon, N. a, Yun, J., & Kester, M. (2000). Ceramide directly activates protein kinase C zeta to regulate a stress-activated protein kinase signaling complex. *The Journal of Biological Chemistry*, *275*(45), 35617–35623. https://doi.org/10.1074/jbc.M007346200

Bowie, M. B., Mcknight, K. D., Kent, D. G., Mccaffrey, L., Hoodless, P. A., & Eaves, C. J. (2006). Hematopoietic stem cells proliferate until after birth and show a reversible phase-specific engraftment defect. *The Journal of Clinical Investigation*, *116*(10), 2808–2816. https://doi.org/10.1172/JCI28310.2808

Bradbury, D. A., Aldington, S., Zhu, Y.-M., & Russell, N. H. (1996). Down-regulation of bcl-2 in AML blasts by all-trans retinoic acid and its relationship to CD34 antigen expression. *British Journal of Haematology*, *94*(4), 671–675. https://doi.org/10.1046/j.1365-2141.1996.d01-1838.x

Brasier, A. R. (2010). The nuclear factor-B-interleukin-6 signalling pathway mediating vascular inflammation. *Cardiovascular Research*, *86*(2), 211–218. https://doi.org/10.1093/cvr/cvq076

Brungs, M., Radmark, O., Samuelsson, B., & Steinhilber, D. (1994). On the Induction of 5-Lipoxygenase Expression and Activity in HL-60 cells: Effects of Vitamin D3, Retinoic Acid and TGFb. *Biochemical and Biophysical Research Communications*, *205*, 1572–1580.

Brungs, M., Radmarkt, O., Samuelssont, B., & Steinhilber, D. (1995). Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor (3 and 1,25-dihydroxyvitamin D3. *Biochemistry*, *92*(January), 107–111. https://doi.org/10.1073/pnas.92.1.107

Büchner, T., Berdel, W. E., Haferlach, C., Haferlach, T., Schnittger, S., Müller-Tidow, C., … Hiddemann, W. (2009). Age-related risk profile and chemotherapy dose response in acute myeloid leukemia: A study by the german acute myeloid leukemia cooperative group. *Journal of Clinical Oncology*, *27*(1), 61–69. https://doi.org/10.1200/JCO.2007.15.4245

Burnett, a. K., Hills, R. K., Milligan, D. W., Goldstone, a. H., Prentice, a. G., McMullin, M.-F., … Wheatley, K. (2009). Attempts to Optimize Induction and Consolidation Treatment in Acute Myeloid Leukemia: Results of the MRC AML12 Trial. *Journal of Clinical Oncology*, *28*(4), 586–595. https://doi.org/10.1200/JCO.2009.22.9088

Busque, L., Patel, J. P., Figueroa, M. E., Vasanthakumar, A., Provost, S., Hamilou, Z., … Levine, R. L. (2012). Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. *Nature Genetics*, *44*(11), 1179–81. https://doi.org/10.1038/ng.2413

Callaghan, R., Luk, F., & Bebawy, M. (2014). Special Section on Transporters in Toxicity and Disease — Minireview Inhibition of the Multidrug Resistance P-Glycoprotein : Time for a Change of Strategy ? *Drug Metabolism and Disposition*, *42*, 623–631.

Campos, L., Rouault, J., Sabido, O., Oriol, P., Roubi, N., Vasselon, C., … Guyotat, D. (1993). High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Blood*, *81*, 3091–3096.

Carter, G., Young, P., Albert, D., Bouska, J., Dyer, R., Bell, R., … Brooks, D. (1991). 5-Lipoxygenase Inhibitory Activity of Zileuton. *The Journal of Pharmacology and Experimental Therapeutics*, *256*(3), 929–937.

Cashen, A. F., Schiller, G. J., O'Donnell, M. R., & DiPersio, J. F. (2010). Multicenter, phase II study of decitabine for the first-line treatment of older patients with Acute Myeloid Leukemia. *Journal of Clinical Oncology*, *28*(4), 556–561. https://doi.org/10.1200/JCO.2009.23.9178

Cassileth, P. a, Lynch, E., Hines, J. D., Oken, M. M., Mazza, J. J., Bennett, J. M., … O'Connell, M. J. (1992). Varying intensity of postremission therapy in acute myeloid leukemia. *Blood*, *79*(8), 1924–30.

Challen, G. a, Sun, D., Jeong, M., Luo, M., Jelinek, J., Berg, J. S., … Goodell, M. a. (2012). Dnmt3a is essential for hematopoietic stem cell differentiation. *Nature Genetics*, *44*(1), 23–31. https://doi.org/10.1038/ng.1009

Chen, L. Y., Eberlein, M., Alsaaty, S., Martinez-Anton, A., Barb, J., Munson, P. J., … Woszczek, G. (2011). Cooperative and redundant signaling of leukotriene B4 and leukotriene D4 in human monocytes. *Allergy*, *66*(10), 1304–11. https://doi.org/10.1111/j.1398-9995.2011.02647.x

Chen, S.-J., & Zhou, G.-B. (2012). Targeted therapy: The new lease on life for acute promyelocytic leukemia, and beyond. *IUBMB Life*, *64*(August), 671–5. https://doi.org/10.1002/iub.1055

Chen, X.-S., Sheller, J., Johnson, E., & Funk, C. (1994). Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. *Nature*, *372*, 179–182. https://doi.org/10.1017/CBO9781107415324.004

Chen, X., Eksioglu, E. a, Zhou, J., Zhang, L., Djeu, J., Fortenbery, N., … Wei, S. (2013). Induction of myelodysplasia by myeloid- derived suppressor cells. *Journal of Clinical Investigation*, *123*(November), 21–23. https://doi.org/10.1172/JCI67580.miRNA-146a

Chen, X., Li, N., Wang, S., Wu, N., Hong, J., Jiao, X., … Yang, C. S. (2003). Leukotriene A 4 Hydrolase in Rat and Human Esophageal Adenocarcinomas and Inhibitory Effects of Bestatin. *Journal of the National Cancer Institute*, *95*(14), 1053–1061.

Chen, Y., Hu, Y., Zhang, H., Peng, C., & Li, S. (2009). Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. *Nature Genetics*, *41*(7), 783–92. https://doi.org/10.1038/ng.389

Chen, Y., Li, D., & Li, S. (2009). The Alox5 gene is a novel therapeutic target in cancer stem cells of chronic myeloid leukemia. *Cell Cycle*, *8*(21), 3488–3492.

Chen, Y., Shan, Y., Lu, M., DeSouza, N., Guo, Z., Hoffman, R., … Li, S. (2016). Alox5 Blockade Eradicates JAK2V617F-Induced Polycythemia Vera in Mice. *Cancer Research*, 1–11. https://doi.org/10.1158/0008-5472.CAN-15-2933

Chen, Y., Sullivan, C., Peng, C., Shan, Y., Hu, Y., Li, D., & Li, S. (2011). A tumor suppressor function of the Msr1 gene in leukemia stem cells of chronic myeloid leukemia. *Blood*, *118*, 390–400. https://doi.org/10.1182/blood-2010-11-316760

Cheshier, S. H., Morrison, S. J., Liao, X., & Weissman, I. L. (1999). In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(6), 3120–5.

Cheson, B. D., Bennett, J. M., Kopecky, K. J., Büchner, T., Willman, C. L., Estey, E. H., … Bloomfield, C. D. (2003). Revised Recommendations of the International Working Group for diagnosis, standardization of response criteria, treatment outcomes, and reporting standards for therapeutic trials in acute myeloid leukemia. *Journal of Clinical Oncology*, *21*(24), 4642–4649. https://doi.org/10.1200/JCO.2003.04.036

Chotinantakul, K., & Leeanansaksiri, W. (2012). Hematopoietic stem cell development, niches, and signaling pathways. *Bone Marrow Research*, *2012*, 1–16. https://doi.org/10.1155/2012/270425

Chung, J., Kim, G., Mun, Y., & Ahn, J. (2005). Leukotriene B4 pathway regulates the fate of the hematopoietic stem cells. *Experimental & Molecular Medicine*, *37*(February 2005), 45–50.

Claesson, H. E., Dahlberg, N., & Gahrton, G. (1985). Stimulation of human myelopoiesis by leukotriene B4. *Biochemical and Biophysical Research Communications*, *131*, 579–585. https://doi.org/10.1016/0006-291X(85)91276-8

Colmone, A., Amorim, M., Pontier, A. L., Wang, S., Jablonski, E., & Sipkins, D. a. (2008). Leukemic Cells Create Bone Marrow Niches That Disrupt the Behavior of Normal Hematopoietic Progenitor Cells. *Science*, *322*, 1861–1865.

Corey, E. J. (1982). Chemical studies on slow reacting substances/leukotrienes. *Experientia*. https://doi.org/10.1007/BF01954913

Costello, R. t, Mallet, F., Gaugler, B., Sainty, D., Arnoulet, C., Gastaut, J., & Olive, D. (2000). Human Acute Myeloid Leukemia CD34 /CD38 Progenitor Cells Have Decreased Sensitivity to Chemotherapy and Fas-induced Apoptosis, Reduced Immunogenicity, and Impaired Dendritic Cell Transformation Capacities. *Cancer Research*, *60*, 4403–4411.

Cuenco, G. M., & Ren, R. (2001). Cooperation of BCR-ABL and AML1/MDS1/EVI1 in blocking myeloid differentiation and rapid induction of an acute myelogenous leukemia. *Oncogene*, *20*(57), 8236–48. https://doi.org/10.1038/sj.onc.1205095

Cursiefen, C., Maruyama, K., Bock, F., Saban, D., Sadrai, Z., Lawler, J., … Masli, S. (2011). Thrombospondin 1 inhibits inflammatory lymphangiogenesis by CD36 ligation on monocytes. *The Journal of Experimental Medicine*, *208*, 1083–1092. https://doi.org/10.1084/jem.20092277

Dash, A., & Gilliland, D. G. (2001). Molecular genetics of acute myeloid leukaemia. *Best Practice & Research. Clinical Haematology*, *14*(1), 49–64. https://doi.org/10.1053/beha.2000.0115

de Groot, D. J. A., de Vries, E. G. E., Groen, H. J. M., & de Jong, S. (2007). Non-steroidal anti-inflammatory drugs to potentiate chemotherapy effects: From lab to clinic. *Critical Reviews in Oncology/Hematology*, *61*(1), 52–69. https://doi.org/10.1016/j.critrevonc.2006.07.001

De Kouchkovsky, I., & Abdul-Hay, M. (2016). "Acute myeloid leukemia: a comprehensive review and 2016 update." *Blood Cancer Journal*, *6*(7), e441. https://doi.org/10.1038/bcj.2016.50

Dean, M., Fojo, T., & Bates, S. (2005). Tumour stem cells and drug resistance. *Nat Rev Cancer*, *5*(4), 275–284. https://doi.org/10.1038/nrc1590

de Sauvage, F. J., Carver-Moore, K., Luoh, S. M., Ryan, A., Dowd, M., Eaton, D. L., & Moore, M. W. (1996). Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. *The Journal of Experimental Medicine*, *183*(2), 651–6.

de Visser, K. E., & Jonkers, J. (2009). Towards understanding the role of cancer-associated inflammation in chemoresistance. *Curr Pharm Des*, *15*(16), 1844–1853. https://doi.org/10.2174/138161209788453239

DeKelver, R. C., Lewin, B., Lam, K., Komeno, Y., Yan, M., Rundle, C., … Zhang, D.-E. (2013). Cooperation between RUNX1-ETO9a and novel transcriptional partner KLF6 in upregulation of Alox5 in acute myeloid leukemia. *PLoS Genetics*, *9*(10), e1003765. https://doi.org/10.1371/journal.pgen.1003765

Delia, D., Aiello, A., Soligo, D., Fontanella, E., Melani, C., Pezzella, F., … Della Porta, G. (1992). Bcl-2 Proto-Oncogene Expression in Normal and Neoplastic Human Myeloid Cells. *Blood*, *79*(5), 1291–1298.

Dick, J. E. (2003). Stem cells: Self-renewal writ in blood. *Nature*, *423*(6937), 231–3. https://doi.org/10.1038/423231a

Dierks, C., Beigi, R., Guo, G. R., Zirlik, K., Stegert, M. R., Manley, P., … Warmuth, M. (2008). Expansion of Bcr-Abl-Positive Leukemic Stem Cells Is Dependent on Hedgehog Pathway Activation. *Cancer Cell*, *14*(3), 238–249. https://doi.org/10.1016/j.ccr.2008.08.003

Ding, L., & Morrison, S. J. (2013). Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature*, *495*(7440), 231–5. https://doi.org/10.1038/nature11885

Ding, L., Saunders, T. L., Enikolopov, G., & Morrison, S. J. (2012). Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature*, *481*(7382), 457–62. https://doi.org/10.1038/nature10783

Döhner, H., Estey, E. H. E., Amadori, S., Appelbaum, F. R. F. R., Büchner, T., Burnett, A. K. a. K., … Others. (2010). Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood*, *115*(3), 453–474. https://doi.org/10.1182/blood-2009-07-235358.

Doulatov, S., Notta, F., Laurenti, E., & Dick, J. E. (2012). Hematopoiesis: A human perspective. *Cell Stem Cell*, *10*(2), 120–136. https://doi.org/10.1016/j.stem.2012.01.006

Drazen, J. M., Silverman, E. K., & Lee, T. H. (2000). asthma, *56*(i), 1054–1070.

Drolle, H., Wagner, M., Vasold, J., K??tt, A., Deniffel, C., Sotlar, K., … Fiegl, M. (2014). Hypoxia regulates proliferation of acute myeloid leukemia and sensitivity against chemotherapy. *Leukemia Research*, *39*(7), 779–785. https://doi.org/10.1016/j.leukres.2015.04.019

Dubois, R. N., Abramson, S. B., Crofford, L., Gupta, R. A., Simon, L. S., Van De Putte, L. B., & Lipsky, P. E. (1998). Cyclooxygenase in biology and disease. *The FASEB Journal*, *12*(12), 1063–1073. https://doi.org/9737710

Dührsen, U., & Hossfeld, D. K. (1996). Stromal abnormalities in neoplastic bone marrow diseases. *Annals of Hematology*, *73*(2), 53–70. https://doi.org/10.1007/s002770050203

Dumoulin, M., Johnson, R. J. K., Bellotti, V., & Dobson, C. M. (1977). Human Lysozyme. In *Protein Misfolding, Aggregation, and Conformational Diseases* (Vol. 6, pp. 285–308).

Eaton, A., Nagy, E., Pacault, M., Fauconnier, J., & Bäck, M. (2012). Cysteinyl leukotriene signaling through perinuclear CysLT1 receptors on vascular smooth muscle cells transduces nuclear calcium signaling and alterations of gene expression. *Journal of Molecular Medicine*, *90*(2012), 1223–1231. https://doi.org/10.1007/s00109-012-0904-1

Ehninger, A., & Trumpp, A. (2011). The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. *The Journal of Experimental Medicine*, *208*(3), 421–8. https://doi.org/10.1084/jem.20110132

Eisele, G., Roth, P., Hasenbach, K., Aulwurm, S., Wolpert, F., Tabatabai, G., … Weller, M. (2011). APO010, a synthetic hexameric CD95 ligand, induces human glioma cell death in vitro and in vivo. *Neuro-Oncology*, *13*(2), 155–164. https://doi.org/10.1093/neuonc/noq176

El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., … Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell*, *75*(4), 817–825. https://doi.org/10.1016/0092-8674(93)90500-P

Eppert, K., Takenaka, K., Lechman, E. R., Waldron, L., Nilsson, B., van Galen, P., … Dick, J. E. (2011). Stem cell gene expression programs influence clinical outcome in human leukemia. *Nature Medicine*, *17*(9), 1086–1093. https://doi.org/10.1038/nm.2415

Estey, E. (2009). Treatment of acute myeloid leukemia. *Haematologica*, *94*(1), 10–16. https://doi.org/10.3324/haematol.2008.001263

Estey, E., Levine, R. L., & Bob, L. (2015). Current challenges in clinical development of " targeted therapies " : the case of acute myeloid leukemia. *Blood*, *125*(16), 2461–2466. https://doi.org/10.1182/blood-2015-01-561373.

Eyberger, A. L., Dondapati, R., & Porter, J. R. (2006). Rapid Communications. *Communications*, *69*(8), 8–11.

Febbraio, M., Abumrad, N. A., David, P., Sharma, K., Cheng, W., Pearce, F. a, … Pearce, S. F. a. (1999). A Null Mutation in Murine CD36 Reveals an Important Role in Fatty Acid and Lipoprotein Metabolism. *The Journal of Biological Chemistry*, *274*(27), 19055–19062. https://doi.org/10.1074/jbc.274.27.19055

Febbraio, M., Hajjar, D. P., & Silverstein, R. L. (2001). CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis,inflammation, and lipid metabolism. *J.Clin.Invest.*, *108*(6), 785–791. https://doi.org/10.1172/JCI200114006.CD36

Fenaux, P., Mufti, G. J., Hellström-Lindberg, E., Santini, V., Gattermann, N., Germing, U., … Silverman, L. R. (2010). Azacitidine prolongs overall survival compared with conventional care regimens in elderly patients with low bone marrow blast count acute myeloid leukemia. *Journal of Clinical Oncology*, *28*(4), 562–569. https://doi.org/10.1200/JCO.2009.23.8329

Feuring-Buske, M., Frankel, A. E., Alexander, R. L., Gerhard, B., & Hogge, D. E. (2002). A diphtheria toxin-interleukin 3 fusion protein is cytotoxic to primitive acute myeloid leukemia progenitors but spares normal progenitors. *Cancer Research*, *62*(6), 1730–1736.

Fialkow, P. J., Gartler, S. M., & Yoshida, A. (1967). Clonal origin of chronic myelocytic leukemia in man. *Proceedings of the National Academy of Sciences of the United States of America*, *58*(4), 1468–71. https://doi.org/10.1073/pnas.58.4.1468

Foell, D., & Roth, J. (2004). Proinflammatory S100 proteins in arthritis and autoimmune disease. *Arthritis and Rheumatism*, *50*(12), 3762–3771. https://doi.org/10.1002/art.20631

Folco, G., & Murphy, R. C. (2006). Eicosanoid Transcellular Biosynthesis: From Cell-Cell Interactions to in Vivo Tissue Responses. *Pharmacological Reviews*, *58*(3), 375–388. https://doi.org/10.1124/pr.58.3.8.375

Geiger, H., de Haan, G., & Florian, M. C. (2013). The ageing haematopoietic stem cell compartment. *Nature Reviews. Immunology*, *13*(5), 376–89. https://doi.org/10.1038/nri3433

Gentles, A. J., Plevritis, S. K., & Page, P. (2012). Association of a Leukemic Stem Cell Gene in Acute Myeloid Leukemia. *JAMA : The Journal of the American Medical Association*, *304*(24).

Georgieva, M., Krasteva, M., Angelova, E., Ralchev, K., Dimitrov, V., Bozhimirov, S., … Berger, M. R. (2008). Analysis of the K-ras/B-raf/Erk signal cascade, p53 and CMAP as markers for tumor progression in colorectal cancer patients. *Oncology Reports*, *20*, 3–11.

Ghoshal, S., Baumann, H., & Wetzler, M. (2008). Epigenetic Regulation of Signal Transducer and Activator of Transcription 3 in Acute Myeloid Leukemia. *Leukemia Research*, *32*(7), 1005–1014. https://doi.org/10.1002/aur.1474.Replication

Gianni, L., Herman, E. H., Lipshultz, S. E., Minotti, G., Sarvazyan, N., & Sawyer, D. B. (2008). Anthracycline cardiotoxicity: From bench to bedside. *Journal of Clinical Oncology*, *26*(22), 3777–3784. https://doi.org/10.1200/JCO.2007.14.9401

Gilliland, D. G., & Tallman, M. S. (2002). Focus on acute leukemias. *Cancer Cell*, *1*(5), 417–420. https://doi.org/10.1016/S1535-6108(02)00081-8

Gottesman, M. M., Fojo, T., & Bates, S. E. (2002). Multidrug Resistance in Cancer: Role of Atp-Dependent Transporters. *Nature Reviews Cancer*, *2*(1), 48–58. https://doi.org/10.1038/nrc706

Graham, S. M., Jørgensen, H. G., Allan, E., Pearson, C., Alcorn, M. J., Richmond, L., & Holyoake, T. L. (2002). Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. *Blood*, *99*(1), 319–325. https://doi.org/10.1182/blood.V99.1.319

Grimwade, D., & Freeman, S. D. (2016). Defining minimal residual disease in acute myeloid leukemia: which platforms are ready for “prime time”?, *124*(23), 222–233. https://doi.org/10.1182/blood-2014-05-577593.This

Grimwade, D., Walker, H., Harrison, G., Oliver, F., Chatters, S., Harrison, C. J., … Burnett, A. K. (2001). The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the. *Blood*, *98*(5), 1312–1321.

Grimwade, D., Walker, H., Oliver, F., Wheatley, K., Harrison, C., Harrison, G., … Goldstone, A. (1998). The Importance of Diagnostic Cytogenetics on Outcome in AML: Analysis of 1,612 Patients Entered Into the MRC AML 10 Trial. *Blood*, *92*(7), 2322–2333.

Guan, Y., Gerhard, B., & Hogge, D. E. (2003). Detection, isolation, and stimulation of quiescent primitive leukemic progenitor cells from patients with acute myeloid leukemia (AML). *Blood*, *101*(8), 3142. https://doi.org/10.1182/blood-2002-10-3062.Supported

Guryanova, O. A., Shank, K., Spitzer, B., Luciani, L., Koche, R. P., Garrett-bakelman, F. E., … Nimer, S. D. (n.d.). DNMT3A mutations promote anthracycline resistance in acute myeloid leukemia via impaired nucleosome remodeling. https://doi.org/10.1038/nm.4210

Guryanova, O. A., Shank, K., Spitzer, B., Luciani, L., Koche, R. P., Garrett-bakelman, F. E., … Nimer, S. D. (2016). DNMT3A mutations promote anthracycline resistance in acute myeloid leukemia via impaired nucleosome remodeling. *Nature Medicine*, *22*(12), 1488–1495. https://doi.org/10.1038/nm.4210

Guzman, M. L., Neering, S. J., Upchurch, D., Grimes, B., Howard, D. S., Rizzieri, D. a, … Jordan, C. T. (2001). Nuclear factor- kB is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood*, *98*(8), 2301–2308. https://doi.org/10.1182/blood.V98.8.2301

Guzman, M. L., Rossi, R. M., Karnischky, L., Li, X., Peterson, D. R., Howard, D. S., & Jordan, C. T. (2005). The sesquiterpene lactone parthenolide induces apoptosis of human acute myelogenous leukemia stem and progenitor cells. *Blood*, *105*(11), 4163–4169. https://doi.org/10.1182/blood-2004-10-4135.Supported

Guzman, M. L., Swiderski, C. F., Howard, D. S., Grimes, B. A., Rossi, R. M., Szilvassy, S. J., & Jordan, C. T. (2002). Preferential induction of apoptosis for primary human leukemic stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(25), 16220–5. https://doi.org/10.1073/pnas.252462599

Hamilton, G., Colbert, J., Schuettelkopf, A., & Watts, C. (2008). Cystatin F is a cathepsin C-directed protease inhibitor regulated by proteolysis. *The EMBO Journal*, *27*(3), 499–508. https://doi.org/10.1038/sj.emboj.7601979

Han, J., Hajjar, D. P., Febbraio, M., & Nicholson, A. C. (1997). Native and Modified Low Density Lipoproteins Increase the Functional Expression of the Macrophage Class B Scavenger Receptor , CD36. *The Journal of Biological Chemistry*, *272*(34), 21654–21659.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, *100*(1), 57–70. https://doi.org/10.1007/s00262-010-0968-0

Hartmann, T. N., Burger, J. a, Glodek, A., Fujii, N., & Burger, M. (2005). CXCR4 chemokine receptor and integrin signaling co-operate in mediating adhesion and chemoresistance in small cell lung cancer (SCLC) cells. *Oncogene*, *24*(27), 4462–4471. https://doi.org/10.1038/sj.onc.1208621

Heidel, F. H., Mar, B. G., & Armstrong, S. A. (2011). Self-renewal related signaling in myeloid leukemia stem cells. *International Journal of Hematology*, *94*(2), 109–117. https://doi.org/10.1007/s12185-011-0901-0

Hessian, P. A., Edgeworth, J., & Hogg, N. (1993). MRP-8 and MRP-14, two abundant CA2+-binding proteins of neutrophils and monocytes. *Cancer Research*, *53*(February), 197–204.

Ho, T.-C., LaMere, M., Stevens, B. M., Ashton, J. M., Myers, J. R., O'Dwyer, K. M., … Becker, M. W. (2016). Evolution of acute myelogenous leukemia stem cell properties following treatment and progression. *Blood*. https://doi.org/10.1182/blood-2016-02-695312

Hoey, T., Yen, W. C., Axelrod, F., Basi, J., Donigian, L., Dylla, S., … Gurney, A. (2009). DLL4 Blockade Inhibits Tumor Growth and Reduces Tumor-Initiating Cell Frequency. *Cell Stem Cell*, *5*(2), 168–177. https://doi.org/10.1016/j.stem.2009.05.019

Horton, S. J., & Huntly, B. J. P. (2012). Recent advances in acute myeloid leukemia stem cell biology. *Haematologica*, *97*(7), 966–974. https://doi.org/10.3324/haematol.2011.054734

Hosen, N., Park, C. Y., Tatsumi, N., Oji, Y., Sugiyama, H., Gramatzki, M., … Weissman, I. L. (2007). CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia. *Proc Natl Acad Sci U S A*, *104*(26), 11008–13. https://doi.org/10.1073/pnas.0704271104

Hu, Y., & Smyth, G. K. (2009). ELDA: Extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *Journal of Immunological Methods*, *347*(1–2), 70–78. https://doi.org/10.1016/j.jim.2009.06.008

Ishii, S., Noguchi, M., Miyano, M., Matsumoto, T., & Noma, M. (1992). Mutagenesis studies on the amino acid residues involved in the iron-binding and the activity of human 5-lipoxygenase. *Biochemical and Biophysical Research Communications*, *182*, 1482–1490. https://doi.org/10.1016/0006-291X(92)91901-2

Ishikawa, F., Yoshida, S., Saito, Y., Hijikata, A., Kitamura, H., Tanaka, S., … Shultz, L. D. (2007). Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nature Biotechnology*, *25*(11), 1315–1321. https://doi.org/10.1038/nbt1350

Ito, T., Kwon, H. Y., Zimdahl, B., Congdon, K. L., Blum, J., Lento, W. E., … Reya, T. (2010). Regulation of myeloid leukaemia by the cell-fate determinant Musashi. *Nature*, *466*(7307), 765–8. https://doi.org/10.1038/nature09171

Ivanova, N. B., Dimos, J. T., Schaniel, C., Hackney, J. A., Moore, K. A., & Lemischka, I. R. (2002). A Stem Cell Molecular Signature. *Science*, *298*(5593), 601–604. https://doi.org/10.1126/science.1073823

Ji, H., Ehrlich, L. I. R., Seita, J., Murakami, P., Doi, A., Lindau, P., … Feinberg, A. P. (2010). Comprehensive methylome map of lineage commitment from haematopoietic progenitors. *Nature*, *467*(7313), 338–42. https://doi.org/10.1038/nature09367

Jin, L., Hope, K. J., Zhai, Q., Smadja-Joffe, F., & Dick, J. E. (2006). Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nature Medicine*, *12*(10), 1167–1174. https://doi.org/10.1038/nm1483

Johns, J. L., & Christopher, M. M. (2012). Extramedullary hematopoiesis: a new look at the underlying stem cell niche, theories of development, and occurrence in animals. *Veterinary Pathology*, *49*(3), 508–23. https://doi.org/10.1177/0300985811432344

Jordan, C. T. (2002). Unique molecular and cellular features of acute myelogenous leukemia stem cells. *Leukemia*, *16*(4), 559–562. https://doi.org/10.1038/sj/leu/2402446

Jordan, C. T., Guzman, M. L., & Noble, M. (2006). Cancer stem cells. *The New England Journal of Medicine*, *355*, 1253–1261. https://doi.org/10.1056/NEJMra061808

Jordan, C. T., Upchurch, D., Szilvassy, S. J., Guzman, M. L., Howard, D. S., Pettigrew, a L., … Phillips, G. L. (2000). The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leukemia*, *14*(10), 1777–1784. https://doi.org/10.1038/sj.leu.2401903

Juliano, R. L., & Ling, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochemica et Biophysica Acta*, *455*(1), 152–162. https://doi.org/10.1016/0005-2736(76)90160-7

Juliusson, G., Antunovic, P., Derolf, Å., Lehmann, S., Möllgård, L., Stockelberg, D., … Höglund, M. (2014). Age and acute myeloid leukemia : real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry Age and acute myeloid leukemia : real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry. *Blood*, *113*(18), 4179–4187. https://doi.org/10.1182/blood-2008-07-172007

Karp, J. (2011). Acute myeloid leukemia. *Leukemia & Lymphoma Society:*, 1–52.

Kell, W. J., Burnett, A. K., Chopra, R., Yin, J. a L., Clark, R. E., Rohatiner, A., … Milligan, D. W. (2003). A feasibility study of simultaneous administration of gemtuzumab ozogamicin with intensive chemotherapy in induction and consolidation in younger patients with acute myeloid leukemia. *Blood*, *102*(13), 4277–4283. https://doi.org/10.1182/blood-2003-05-1620.Supported

Kharas, M. G., Lengner, C. J., Al-Shahrour, F., Bullinger, L., Ball, B., Zaidi, S., … Daley, G. Q. (2010). Musashi-2 regulates normal hematopoiesis and promotes aggressive myeloid leukemia. *Nature Medicine*, *16*(8), 903–8. https://doi.org/10.1038/nm.2187

Kharbanda, S., Nakamura, T., Stone, R., Hass, R., Bernstein, S., Datta, R., … Kufe, D. (1991). Expression of the early growth response 1 and 2 zinc finger genes during induction of monocytic differentiation. *The Journal of Clinical Investigation*, *88*(2), 571–7. https://doi.org/10.1172/JCI115341

Kiel, M. J., & Morrison, S. J. (2008). Uncertainty in the niches that maintain haematopoietic stem cells. *Nature Reviews. Immunology*, *8*(4), 290–301. https://doi.org/10.1038/nri2279

Kiel, M. J., Yilmaz, O. H., Iwashita, T., Yilmaz, O. H., Terhorst, C., & Morrison, S. J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell*, *121*(7), 1109–21. https://doi.org/10.1016/j.cell.2005.05.026

Kihara, R., Nagata, Y., Kiyoi, H., Kato, T., Yamamoto, E., Suzuki, K., … Naoe, T. (2014). Comprehensive analysis of genetic alterations and their prognostic impacts in adult acute myeloid leukemia patients. *Leukemia*, *28*(8), 1586–1595. https://doi.org/10.1038/leu.2014.55

Kimura, S., Roberts, a W., Metcalf, D., & Alexander, W. S. (1998). Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(3), 1195–200.

Klampfer, L., Zhang, J., Zelenetz, A. O., Uchida, H., & Nimer, S. D. (1996). The AML1/ETO fusion protein activates transcription of BCL-2. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(24), 14059–64.

Konopleva, M., Zhao, S., Hu, W., Jiang, S., Snell, V., Weidner, D., … Andreeff, M. (2002). The anti-apoptotic genes Bcl-XL and Bcl-2 are over-expressed and contribute to chemoresistance of non-proliferating leukaemic CD34+ cells. *British Journal of Haematology*, *118*(2), 521–534. https://doi.org/10.1046/j.1365-2141.2002.03637.x

Krivtsov, A. V, Twomey, D., Feng, Z., Stubbs, M. C., Wang, Y., Faber, J., … Armstrong, S. a. (2006). Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature*, *442*(7104), 818–22. https://doi.org/10.1038/nature04980

Kurtova, A. V., Xiao, J., Mo, Q., Pazhanisamy, S., Krasnow, R., Lerner, S. P., … Chan, K. S. (2014). Blocking PGE2-induced tumour repopulation abrogates bladder cancer chemoresistance. *Nature*, *517*(7533), 209–213. https://doi.org/10.1038/nature14034

Lagadinou, E. D., Sach, A., Callahan, K., Rossi, R. M., Neering, S. J., Minhajuddin, M., … Jordan, C. T. (2013). BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. *Cell Stem Cell*, *12*(3), 329–341. https://doi.org/10.1016/j.stem.2012.12.013

Lancet, J. E., Uy, G. L., Cortes, J. E., Newell, L. F., Lin, T. L., Ritchie, E. K., … Medeiros, B. C. (2016). Final results of a phase III randomized trial of CPX-351 versus 7+3 in older patients with newly diagnosed high risk (secondary) AML. In *ASCO Annual Meeting* (p. 7000).

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., … Dick, J. E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature*, *367*(6464), 645–648. https://doi.org/10.1038/367645a0

Laurent, G., Jaffrézou, J., & Jaffre, J. (2001). Signaling pathways activated by daunorubicin. *Blood*, *98*(4), 913–924. https://doi.org/10.1182/blood.V98.4.913

Lavau, C., Szilvassy, S. J., Slany, R., & Cleary, M. L. (1997). Immortalization and leukemic transformation of a myelomonocytic precursor by retrovirally transduced HRX-ENL. *EMBO Journal*, *16*(14), 4226–4237. https://doi.org/10.1093/emboj/16.14.4226

Lawrence, H. J., Sauvageau, G., Humphries, R. K., & Largman, C. (1996). The role of HOX homeobox genes in normal and leukemic hematopoiesis. *Stem Cells*, *14*(3), 281–291. https://doi.org/10.1002/stem.140281

Legras, S., Gunthert, U., Stauder, R., Curt, F., Oliferenko, S., Kluin-Nelemans, H. C., … Smadja-Joffe, F. (1998). A Strong Expression of CD44-6v Correlates With Shorter Survival of Patients With Acute Myeloid Leukemia. *Blood*, *91*(9), 3401–3414.

Leith, C. P., Kopecky, K. J., Godwin, J., McConnell, T., Slovak, M. L., Chen, I. M., … Willman, C. L. (1997). Acute myeloid leukemia in the elderly: assessment of multidrug resistance (MDR1) and cytogenetics distinguishes biologic subgroups with remarkably distinct responses to standard chemotherapy. A Southwest Oncology Group study. *Blood*, *89*(9), 3323–9.

Leseux, L., Laurent, G., Laurent, C., Rigo, M., Blanc, A., Olive, D., & Bezombes, C. (2008). PKC zeta mTOR pathway: a new target for rituximab therapy in follicular lymphoma. *Blood*, *111*(1), 285–291. https://doi.org/blood-2007-04-085092 [pii]\n10.1182/blood-2007-04-085092

Lessard, J., & Sauvageau, G. (2003). Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature*, *423*(6937), 255–260. https://doi.org/10.1038/nature01572

Li, J. (2011). Quiescence regulators for hematopoietic stem cell. *Experimental Hematology*, *39*(5), 511–20. https://doi.org/10.1016/j.exphem.2011.01.008

Li, L., & Bhatia, R. (2011). Stem cell quiescence. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *17*(15), 4936–41. https://doi.org/10.1158/1078-0432.CCR-10-1499

Liang, C. P., Han, S., Okamoto, H., Carnemolla, R., Tabas, I., Accili, D., & Tall, A. R. (2004). Increased CD36 protein as a response to defective insulin signaling in macrophages. *Journal of Clinical Investigation*, *113*(5), 764–773. https://doi.org/10.1172/JCI200419528

Lucas, C. M., Harris, R. J., Giannoudis, A., McDonald, E., & Clark, R. E. (2014). Low leukotriene B4 receptor 1 leads to ALOX5 downregulation at diagnosis of chronic myeloid leukemia. *Haematologica*, *99*(11), 1710–1715. https://doi.org/10.3324/haematol.2013.101972

Lundeen, K. a, Sun, B., Karlsson, L., & Fourie, A. M. (2006). Leukotriene B4 receptors BLT1 and BLT2: expression and function in human and murine mast cells. *Journal of Immunology (Baltimore, Md. : 1950)*, *177*, 3439–3447. https://doi.org/10.4049/jimmunol.177.5.3439

Magister, Š., Obermajer, N., Mirković, B., Švajger, U., Renko, M., Softić, A., … Kos, J. (2012). Regulation of cathepsins S and L by cystatin F during maturation of dendritic cells. *European Journal of Cell Biology*, *91*(2012), 391–401. https://doi.org/10.1016/j.ejcb.2012.01.001

Majeti, R., Chao, M. P., Alizadeh, A. A., Pang, W. W., Jaiswal, S., Gibbs, K. D., … Weissman, I. L. (2009). CD47 Is an Adverse Prognostic Factor and Therapeutic Antibody Target on Human Acute Myeloid Leukemia Stem Cells. *Cell*, *138*(2), 286–299. https://doi.org/10.1016/j.cell.2009.05.045

Mak, D. H., Wang, R.-Y., Schober, W. D., Konopleva, M., Cortes, J., Kantarjian, H., … Carter, B. Z. (2012). Activation of apoptosis signaling eliminates CD34+ progenitor cells in blast crisis CML independent of response to tyrosine kinase inhibitors. *Leukemia*, *26*(4), 788–94. https://doi.org/10.1038/leu.2011.285

Marceau, K., Ruttle, P. L., Shirtcliff, E. A., Essex, M. J., Susman, E. J., Studies, A., … Orleans, N. (2014). Declining Rates of Treatment-Related Mortality in Patients with Newly-diagnosed AML Given "Intense" Induction Regimens: A Report from SWOG and MD Anderson. *Leukemia*, *28*(2), 289–292. https://doi.org/10.1002/dev.21214.Developmental

Matsuzaki, Y., Nakayama, K., Nakayama, K., Tomita, T., Isoda, M., Loh, D. Y., & Nakauchi, H. (1997). Role of bcl-2 in the development of lymphoid cells from the hematopoietic stem cell. *Blood*, *89*, 853–862.

Mclellan, M. D., Lamprecht, T., Larson, D. E., Ph, D., Kandoth, C., Payton, J. E., … Wilson, R. K. (2010). Mutations in Acute Myeloid Leukemia, 1–10.

Medeiros, A. I., Sá-Nunes, A., Soares, E. G., Peres, C. M., Silva, C. L., & Faccioli, L. H. (2004). Blockade of Endogenous Leukotrienes Exacerbates Pulmonary Histoplasmosis. *Infection and Immunity*, *72*(3), 1637–1644. https://doi.org/10.1128/IAI.72.3.1637-1644.2004

Medeiros, A. I., Silva, C. L., Malheiro, A., Maffei, C. M., & Faccioli, L. H. (1999). Leukotrienes are involved in leukocyte recruitment induced by live Histoplasma capsulatum or by the beta-glucan present in their cell wall. *British Journal of Pharmacology*, *128*(7), 1529–37. https://doi.org/10.1038/sj.bjp.0702912

Mehrabian, M., Allayee, H., Wong, J., Shih, W., Wang, X. P., Shaposhnik, Z., … Lusis, A. J. (2002). Identification of 5-Lipoxygenase as a major gene contributing to atherosclerosis susceptibility in mice. *Circulation Research*, *91*, 120–126. https://doi.org/10.1161/01.RES.0000028008.99774.7F

Metzeler, K. H., Maharry, K., Radmacher, M. D., Mrózek, K., Margeson, D., Becker, H., … Bloomfield, C. D. (2011). TET2 mutations improve the new European LeukemiaNet risk classification of acute myeloid leukemia: A cancer and leukemia group B study. *Journal of Clinical Oncology*, *29*(10), 1373–1381. https://doi.org/10.1200/JCO.2010.32.7742

Mikkola, H. K. a, & Orkin, S. H. (2006). The journey of developing hematopoietic stem cells. *Development (Cambridge, England)*, *133*(19), 3733–44. https://doi.org/10.1242/dev.02568

Milella, M., Kornblau, S. M., Estrov, Z., Carter, B. Z., Lapillonne, H., Harris, D., … Andreeff, M. (2001). Therapeutic targeting of the MEK / MAPK signal transduction module in acute myeloid leukemia. *Journal of Clinical Investigation*, *108*(6), 851–859. https://doi.org/10.1172/JCI200112807.Introduction

Miyaoka, K., Kuwasako, T., Hirano, K., Nozaki, S., Yamashita, S., & Matsuzawa, Y. (2001). CD36 deficiency associated with insulin resistance Prophylaxis with a nevirapine- containing triple regimen after exposure to HIV-1. *The Lancet*, *357*, 686–687.

Mizuki, M., Fenski, R., Halfter, H., Matsumura, I., Schmidt, R., Müller, C., … Serve, H. (2000). Flt3 mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways. *Blood*, *96*(12), 3907–14. https://doi.org/10.1016/0092-8674(91)90010-v

Mohamed, M., & Sloane, B. (2006). Cysteine cathepsins: multifunctional enzymes in cancer. *Nature Reviews Cancer*, *6*(10), 764–75. https://doi.org/10.1038/nrc1949

Momparler, R. L. (2013). Optimization of cytarabine (ARA-C) therapy for acute myeloid leukemia. *Experimental Hematology & Oncology*, *2*(1), 20. https://doi.org/10.1186/2162-3619-2-20

Moore, M. A., & Warren, D. J. (1987). Synergy of interleukin 1 and granulocyte colony-stimulating factor: in vivo stimulation of stem-cell recovery and hematopoietic regeneration following 5-fluorouracil treatment of mice. *Proceedings of the National Academy of Sciences of the United States of America*, *84*(20), 7134–8. https://doi.org/10.1073/pnas.84.20.7134

Mootha, V. K., Lindgren, C. M., Eriksson, K., Subramanian, A., Sihag, S., Lehar, J., … Groop, L. C. (2003). PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nature Genetics*, *34*(3), 267–273. https://doi.org/10.1038/nn1239

Moscinski, L. C., Kasnic, G., & Saker, A. (1991). The Significance of an Elevated Serum Lysozyme Value in Acute Myelogenous Leukemia with Eosinophilia. *Hematopathology*, *97*(2), 195–201.

Mrózek, K. (2008). Cytogenetic, Molecular Genetic, and Clinical Characteristics of Acute Myeloid Leukemia With a Complex Karyotype. *Seminars in Oncology*, *35*(4), 365–377. https://doi.org/10.1053/j.seminoncol.2008.04.007

Mrózek, K., Heerema, N. A., & Bloomfield, C. D. (2004). Cytogenetics in acute leukemia. *Blood Reviews*, *18*(2), 115–136. https://doi.org/10.1016/S0268-960X(03)00040-7

Ng, S. W. K., Mitchell, A., Kennedy, J. A., Chen, W. C., McLeod, J., Ibrahimova, N., … Wang, J. C. Y. (2016). A 17-gene stemness score for rapid determination of risk in acute leukaemia. *Nature*, *540*(7633), 433–437. https://doi.org/10.1038/nature20598

Nguyen, T., Fealgueyret, J.-P., Abramovitz, M., & Riendeau, D. (1991). Evaluation of the Role of Conserved His and Met Residues among Lipoxygenases by Site-directed Mutagenesis of Recombinant Human 5-Lipoxygenase. *Journal of Biological Chemistry*, *266*(32), 22057–22062. https://doi.org/10.1017/CBO9781107415324.004

Ni, J., Fernandez, M. a., Danielsson, L., Chillakuru, R. a., Zhang, J., Grubb, a., … Abrahamson, M. (1998). Cystatin F Is a Glycosylated Human Low Molecular Weight Cysteine Proteinase Inhibitor. *Journal of Biological Chemistry*, *273*, 24797–24804. https://doi.org/10.1074/jbc.273.38.24797

Niemann, C., Unden, A. B., Lyle, S., Zouboulis, C. C., Toftgård, R., & Watt, F. M. (2003). Indian hedgehog and beta-catenin signaling: role in the sebaceous lineage of normal and neoplastic mammalian epidermis. *Proceedings of the National Academy of Sciences of the United States of America*, *100 Suppl*, 11873–80. https://doi.org/10.1073/pnas.1834202100

NIH. (n.d.). http://www.clinicaltrials.gov.

Niiya, M., Niiya, K., Kiguchi, T., Shibakura, M., Asaumi, N., Shinagawa, K., … Tanimoto, M. (2003). Induction of TNF-??, uPA, IL-8 and MCP-1 by doxorubicin in human lung carcinoma cells. *Cancer Chemotherapy and Pharmacology*, *52*(5), 391–398. https://doi.org/10.1007/s00280-003-0665-1

Ogawa, Y., & Calhoun, W. J. (2006). The role of leukotrienes in airway inflammation. *Journal of Allergy and Clinical Immunology*, *118*(4), 789–798. https://doi.org/10.1016/j.jaci.2006.08.009

Ogbomo, H., Michaelis, M., & Klassert, D. (2008). Resistance to Cytarabine Induces the Up-regulation of NKG2D Ligands and Enhances Natural Killer Cell Lysis of Leukemic Cells. *Neoplasia*, *10*(12), 1402–1410. https://doi.org/10.1593/neo.08972

Olsson, I., Bergh, G., Ehinger, M., & Gullberg, U. (1996). Cell differentiation in acute myeloid leukemia. *European Journal of Haematology*, *57*, 1–16.

Orkin, S. H., & Zon, L. I. (2008). Hematopoiesis: An Evolving Paradigm for Stem Cell Biology. *Cell*, *132*(4), 631–644. https://doi.org/10.1016/j.cell.2008.01.025

Ossenkoppele, G., & Lowenberg, B. (2015). How I treat the older patient with acute myeloid leukemia. *Blood*, *125*(5), 767–774. https://doi.org/10.1182/blood-2014-08-551499.c

Osserman, E. F., & Lawlor, D. P. (1966). Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. *Microbiology*, *124*(May), 921–947.

Passegué, E., Jamieson, C. H. M., Ailles, L. E., & Weissman, I. L. (2003). Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? *Proceedings of the National Academy of Sciences of the United States of America*, *100 Suppl*, 11842–9. https://doi.org/10.1073/pnas.2034201100

Patel, C., Stenke, L., Varma, S., Lindberg, M. L., Bj??rkholm, M., Sj??berg, J., … Gillet, J. P. (2013). Multidrug resistance in relapsed acute myeloid leukemia: Evidence of biological heterogeneity. *Cancer*, *119*(16), 3076–3083. https://doi.org/10.1002/cncr.28098

Patel, J. P., Gönen, M., Figueroa, M. E., Fernandez, H., Sun, Z., Racevskis, J., … L. Levine, R. (2012). Prognostic Relevance of Integrated Genetic Profiling in Acute Myeloid Leukemia. *New England Journal of Medicine*, *366*(12), 1079–1089. https://doi.org/10.1056/NEJMoa1402121

Peng, Z., Xiao, Z., Wang, Y., Liu, P., Cai, Y., Lu, S., … Han, Z. C. (2004). Reversal of P-glycoprotein-mediated multidrug resistance with small interference RNA (siRNA) in leukemia cells. *Cancer Gene Therapy*, *11*(2004), 707–12. https://doi.org/10.1038/sj.cgt.7700738

Peres, C. M., de Paula, L., Medeiros, A. I., Sorgi, C. A., Soares, E. G., Carlos, D., … Faccioli, L. H. (2007). Inhibition of leukotriene biosynthesis abrogates the host control of Mycobacterium tuberculosis. *Microbes and Infection*, *9*(4), 483–489. https://doi.org/10.1016/j.micinf.2007.01.006

Peter, B., & Greenberg, L. (1978). Marrow Adherent Cell Colony-stimulating Activity Production in Acute Myeloid Leukemia. *Blood*, *52*(2), 362–379.

Pham, L. V, Tamayo, A. T., Yoshimura, L. C., Lo, P., & Ford, R. J. (2003). Inhibition of constitutive NF-κB activation in mantle cell lymphoma B cells leads to induction of cell cycle arrest and apoptosis. *Journal of Immunology (Baltimore, Md. : 1950)*, *171*(1), 88–95. https://doi.org/10.4049/jimmunol.171.1.88

Pietras, E. M., Warr, M. R., & Passegué, E. (2011). Cell cycle regulation in hematopoietic stem cells. *The Journal of Cell Biology*, *195*(5), 709–20. https://doi.org/10.1083/jcb.201102131

Piper, P. J., Conroy, D. M., Costello, J. F., Evans, J. M., Green, C. P., Price, F., … Spencer, D. A. (1991). Leukotienes and Inflammatory Lung Disease. *Annals of the New York Academy of Sciences*, 112–119. https://doi.org/10.1196/annals.1304.010

Pirooznia, M., Nagarajan, V., & Deng, Y. (2007). GeneVenn - A web application for comparing gene lists using Venn diagrams. *Bioinformation*, *1*(10), 420–422. https://doi.org/10.6026/97320630001420

Pollyea, D. A., Gutman, J. A., Gore, L., Smith, C. A., & Jordan, C. T. (2014). Targeting acute myeloid leukemia stem cells: A review and principles for the development of clinical trials. *Haematologica*, *99*(8), 1277–1284. https://doi.org/10.3324/haematol.2013.085209

Purton, L. E., & Scadden, D. T. (2007). Limiting Factors in Murine Hematopoietic Stem Cell Assays. *Cell Stem Cell*, *1*(3), 263–270. https://doi.org/10.1016/j.stem.2007.08.016

Qian, H., Buza-Vidas, N., Hyland, C. D., Jensen, C. T., Antonchuk, J., Månsson, R., … Jacobsen, S. E. W. (2007). Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. *Cell Stem Cell*, *1*(6), 671–84. https://doi.org/10.1016/j.stem.2007.10.008

Quigley, G., Wang, A., Ughetto, G., Marel, Gi. van der, van Boom, J. H., & Rich, A. (1980). Molecular structure of an anticancer drug-DNA complex: Daunomycin plus d(CpGpTpApCpG). *Proceedings of the National Academy of Sciences of the United States of America*, *77*(12), 7204–7208.

Radmark, O. (2000). The molecular biology and regulation of 5-Lipoxygenase. *Am. J. Respir. Crit. Care Med.*, *161*, S11–S15.

Ran, D., Schubert, M., Taubert, I., Eckstein, V., Bellos, F., Jauch, A., … Ho, A. D. (2012). Heterogeneity of leukemia stem cell candidates at diagnosis of acute myeloid leukemia and their clinical significance. *Experimental Hematology*, *40*(2), 155–165. https://doi.org/10.1016/j.exphem.2011.10.005

Rauch, P. J., Ellegast, J. M., Widmer, C. C., Fritsch, K., Goede, J. S., Valk, P. J. M., … Manz, M. G. (2016). MPL expression on AML blasts predicts peripheral blood neutropenia and thrombocytopenia. *Blood*, *128*(18), 2253–2257. https://doi.org/10.1182/blood-2016-04-711986.The

Ravandi, F., & Estrov, Z. (2006). Eradication of leukemia stem cells as a new goal of therapy in leukemia. *Clinical Cancer Research*, *12*(2), 340–344. https://doi.org/10.1158/1078-0432.CCR-05-1879

Renneville, A., Roumier, C., Biggio, V., Nibourel, O., Boissel, N., Fenaux, P., & Preudhomme, C. (2008). Cooperating gene mutations in acute myeloid leukemia: a review of the literature. *Leukemia*, *22*(5), 915–31. https://doi.org/10.1038/leu.2008.19

Reya, T., Morrison, S. J., Clarke, M. F., & Weissman, I. L. (2001). Stem Cells, Cancer, and Cancer Stem Cells. *Nature*, *414*(November), 105–111. https://doi.org/10.1007/978-1-60327-933-8

Ries, C., Loher, F., Zang, C., Ismair, M. G., & Petrides, P. E. (1999). Matrix metalloproteinase production by bone marrow mononuclear cells from normal individuals and patients with acute and chronic myeloid leukemia or myelodysplastic syndromes. *Clinical Cancer Research*, *5*(5), 1115–1124.

Rimessi, A., Patergnani, S., Ioannidi, E., & Pinton, P. (2013). Chemoresistance and Cancer-Related Inflammation: Two Hallmarks of Cancer Connected by an Atypical Link, PKCζ. *Frontiers in Oncology*, *3*(September), 232. https://doi.org/10.3389/fonc.2013.00232

Rioux, N., & Castonguay, A. (1998). Inhibitors of lipoxygenase: a new class of cancer chemopreventive agents. *Carcinogenesis*, *19*(8), 1393–1400.

Roboz, G. J. (2011). Novel approaches to the treatment of acute myeloid leukemia. *Hematology*, 43–50.

Roos, J., Oancea, C., Heinssmann, M., Khan, D., Held, H., Kahnt, A. S., … Ruthardt, M. (2014). 5-Lipoxygenase Is a Candidate Target for Therapeutic Management of Stem Cell-Like Cells in Acute Myeloid Leukemia. *Cancer Research*, *74*(12), 5244–5255. https://doi.org/10.1158/0008-5472.CAN-13-3012

Rosenbauer, F., & Tenen, D. G. (2007). Transcription factors in myeloid development: balancing differentiation with transformation. *Nature Reviews. Immunology*, *7*(2), 105–17. https://doi.org/10.1038/nri2024

Rowe, J. M., & Tallman, M. S. (2010). How I treat acute myeloid leukemia. *Blood*, *116*(17), 3147–56. https://doi.org/10.1182/blood-2010-05-260117

Rumpold, H., Salvador, C., Wolf, A. M., Tilg, H., Gastl, G., & Wolf, D. (2007). Knockdown of PgP resensitizes leukemic cells to proteasome inhibitors. *Biochemical and Biophysical Research Communications*, *361*, 549–554. https://doi.org/10.1016/j.bbrc.2007.07.049

Ryckman, C., Vandal, K., Rouleau, P., Talbot, M., & Tessier, P. a. (2003). Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. *Journal of Immunology (Baltimore, Md. : 1950)*, *170*, 3233–3242. https://doi.org/10.4049/jimmunol.170.6.3233

Samuelsson, B. (1983). Leukotrienes - Mediators of Immediate Hypersensitivity Reactions and Inflammation. *Science*, *220*(4597), 568–575.

Santos, C. Dos, Mcdonald, T., Ho, Y. W., Liu, H., Lin, A., Forman, S. J., … Bhatia, R. (2014). The Src and c-Kit kinase inhibitor dasatinib enhances p53- mediated targeting of human acute myeloid leukemia stem cells by chemotherapeutic agents, *122*(11), 1–14. https://doi.org/10.1182/blood-2012-11-466425.Y.-H.K.

Sarry, J., Murphy, K., Perry, R., Sanchez, P. V, Secreto, A., Keefer, C., … Carroll, M. (2011). Human acute myelogenous leukemia stem cells are rare and heterogeneous when assayed in NOD / SCID / IL2R γ c-deficient mice. *J. Clin. Invest.*, *121*(1), 384–295. https://doi.org/10.1172/JCI41495.384

Secatto, A., Rodrigues, L. C., Serezani, C. H., Ramos, S. G., Dias-Baruffi, M., Faccioli, L. H., & Medeiros, A. I. (2012). 5-Lipoxygenase Deficiency Impairs Innate and Adaptive Immune Responses During Fungal Infection. *PLoS ONE*, *7*(March), 1–9. https://doi.org/10.1371/journal.pone.0031701

Sehgal, A. R., Gimotty, P. A., Zhao, J., Hsu, J. M., Daber, R., Morrissette, J. D., … Carroll, M. (2015). DNMT3A mutational status affects the results of dose-escalated induction therapy in acute myelogenous leukemia. *Clinical Cancer Research*, *21*(7), 1614–1620. https://doi.org/10.1158/1078-0432.CCR-14-0327

Seita, J., Ema, H., Ooehara, J., Yamazaki, S., Tadokoro, Y., Yamasaki, A., … Nakauchi, H. (2007). Lnk negatively regulates self-renewal of hematopoietic stem cells by modifying thrombopoietin-mediated signal transduction. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(7), 2349–2354. https://doi.org/10.1073/pnas.0606238104

Seita, J., Sahoo, D., Rossi, D. J., Bhattacharya, D., Serwold, T., Inlay, M. A., … Weissman, I. L. (2012). Gene expression commons: An open platform for absolute gene expression profiling. *PLoS ONE*, *7*(7), 1–11. https://doi.org/10.1371/journal.pone.0040321

Seita, J., & Weissman, I. L. (2010). Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdisciplinary Reviews. Systems Biology and Medicine*, *2*(6), 640–53. https://doi.org/10.1002/wsbm.86

Shih, A. H., Jiang, Y., Meydan, C., Shank, K., Pandey, S., Barreyro, L., … Levine, R. L. (2015). Mutational cooperativity linked to combinatorial epigenetic gain of function in acute myeloid leukemia. *Cancer Cell*, *27*(4), 502–515. https://doi.org/10.1016/j.ccell.2015.03.009

Shlush, L. I., Zandi, S., Mitchell, A., Chen, W. C., Brandwein, J. M., Gupta, V., … Dick, J. E. (2014). Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature*, *506*(7488), 328–333. https://doi.org/10.1038/nature13038

Signore, M., Ricci-Vitiani, L., & De Maria, R. (2013). Targeting apoptosis pathways in cancer stem cells. *Cancer Letters*, *332*(2), 374–382. https://doi.org/10.1016/j.canlet.2011.01.013

Sill, H., Olipitz, W., Zebisch, A., Schulz, E., & Wölfler, A. (2011). Therapy-related myeloid neoplasms: Pathobiology and clinical characteristics. *British Journal of Pharmacology*, *162*(4), 792–805. https://doi.org/10.1111/j.1476-5381.2010.01100.x

Sironi, S., Wagner, M., Kuett, A., Drolle, H., Polzer, H., Spiekermann, K., … Fiegl, M. (2015). Microenvironmental Hypoxia regulates FLT3 expression and biology in AML. *Scientific Reports*, *5*, 17550. https://doi.org/10.1038/srep17550

Sladek, K., Dworski, R., Fitzgerald, G. A., Buitkus, K. L., Block, F. J., Marney, S. R., & Sheller, J. R. (1990). Allergen-stimulated Release of Thromboxane A2 and Leukotriene E4 in Humans. *American Review of Respiratory Disease*, *141*(6), 1441–1445.

Slovak, M. L., Kopecky, K. J., Cassileth, P. A., Harrington, D. H., Theil, K. S., Mohamed, A., … Appelbaum, F. R. (2015). Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia : a Southwest Oncology Group / Eastern Cooperative Oncology Group study. *Blood*, *96*(13), 4075–4084.

Smeets, M. E. P., Raymakers, R. A. P., Vierwinden, G., Pennings, A. H. M., Boezeman, J., Minderman, H., & Witte, T. M. De. (1999). Idarubicin DNA intercalation is reduced by MRP1 and not Pgp. *Leukemia*, *13*, 1390–1398.

Somervaille, T. C. P., & Cleary, M. L. (2006). Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. *Cancer Cell*, *10*(October), 257–268. https://doi.org/10.1016/j.ccr.2006.08.020

Spijkers-Hagelstein, J. a P., Schneider, P., Hulleman, E., de Boer, J., Williams, O., Pieters, R., & Stam, R. W. (2012). Elevated S100A8/S100A9 expression causes glucocorticoid resistance in MLL-rearranged infant acute lymphoblastic leukemia. *Leukemia*, *26*(6), 1255–65. https://doi.org/10.1038/leu.2011.388

Stavropoulou, V., Kaspar, S., Brault, L., Sanders, M. A., Juge, S., Morettini, S., … Schwaller, J. (2016). MLL-AF9 Expression in Hematopoietic Stem Cells Drives a Highly Invasive AML Expressing EMT-Related Genes Linked to Poor Outcome. *Cancer Cell*, *30*(1), 43–58. https://doi.org/10.1016/j.ccell.2016.05.011

Stiehl, T., Baran, N., Ho, A. D., & Marciniak-Czochra, A. (2015). Cell Division Patterns in Acute Myeloid Leukemia Stem-like Cells Determine Clinical Course : A Model to Predict Patient Survival. *Cancer Research*, *75*(6), 940–949. https://doi.org/10.1158/0008-5472.CAN-14-2508

Stoka, V., Turk, B., Schendel, S., & Kim, T. (2001). Lysosomal protease pathways to apoptosis cleavage of Bid, not pro-caspases, is the most likely route. *Journal of Biological ...*, *276*(5), 3149–57. https://doi.org/10.1074/jbc.M008944200

Stone, R. M., DeAngelo, D. J., Klimek, V., Galinsky, I., Estey, E., Nimer, S. D., … Griffin, J. D. (2005). Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. *Blood*, *105*(1), 54–60. https://doi.org/10.1182/blood-2004-03-0891

Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. a, … Mesirov, J. P. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(43), 15545–50. https://doi.org/10.1073/pnas.0506580102

Susa, M., Iyer, A. K., Ryu, K., Choy, E., Hornicek, F. J., Mankin, H., … Duan, Z. (2010). Inhibition of ABCB1 (MDR1) expression by an siRNA nanoparticulate delivery system to overcome drug resistance in osteosarcoma. *PLoS ONE*, *5*(5). https://doi.org/10.1371/journal.pone.0010764

Tager, A. M., Bromley, S. K., Medoff, B. D., Islam, S. A., Bercury, S. D., Friedrich, E. B., … Luster, A. D. (2003). Leukotriene B4 receptor BLT1 mediates early effector T cell recruitment. *Nature Immunology*, *4*(10), 982–990. https://doi.org/10.1038/ni970

Takebayashi, T., Higashi, H., Sudo, H., Ozawa, H., Suzuki, E., Shirado, O., … Hatakeyama, M. (2003). NF-kB-dependent induction of cyclin D1 by retinoblastoma protein (pRB) family proteins and tumor-derived pRB mutants. *Journal of Biological Chemistry*, *278*(17), 14897–14905. https://doi.org/10.1074/jbc.M210849200

Tang, R., Faussat, A.-M., Perrot, J.-Y., Marjanovic, Z., Cohen, S., Storme, T., … Marie, J.-P. (2008). Zosuquidar restores drug sensitivity in P-glycoprotein expressing acute myeloid leukemia (AML). *BMC Cancer*, *8*, 51. https://doi.org/10.1186/1471-2407-8-51

Terwijn, M., Zeijlemaker, W., Kelder, A., Rutten, A. P., Snel, A. N., Scholten, W. J., … Schuurhuis, G. J. (2014). Leukemic stem cell frequency: A strong biomarker for clinical outcome in acute myeloid leukemia. *PLoS ONE*, *9*(9), 7–9. https://doi.org/10.1371/journal.pone.0107587

Thol, F., Schlenk, R. F., Heuser, M., & Ganser, A. (2015). How I treat refractory and early relapsed acute myeloid leukemia. *Blood*, *126*(3), 319–327. https://doi.org/10.1182/blood-2014-10-551911

Thompson, C., Cloutier, A., Bossé, Y., Thivierge, M., Le Gouill, C., Larivée, P., … Rola-Pleszczynski, M. (2006). CysLT1 receptor engagement induces activator protein-1- and NF-κB-dependent IL-8 expression. *American Journal of Respiratory Cell and Molecular Biology*, *35*(6), 697–704. https://doi.org/10.1165/rcmb.2005-0407OC

Thorsteinsdottir, U., Krosl, J., Kroon, E., Haman, A., Hoang, T., & Sauvageau, G. (1999). The oncoprotein E2A-Pbx1a collaborates with Hoxa9 to acutely transform primary bone marrow cells. *Mol Cell Biol*, *19*(9), 6355–6366.

Towatari, M., Iida, H., Tanimoto, M., Iwata, H., Hamaguchi, M., & Saito, H. (1997). Constitutive activation of mitogen-activated protein kinase pathway in acute leukemia cells. *Leukemia*, *11*(4), 479–84.

Travlos, G. S. (2006). Normal structure, function, and histology of the bone marrow. *Toxicologic Pathology*, *34*(5), 548–65. https://doi.org/10.1080/01926230600939856

Ueno, T., Toi, M., & Saji, H. (2000). Significance of Macrophage Chemoattractant Protein-1 in Macrophage Recruitment , Angiogenesis , and Survival in Human Breast Cancer Significance of Macrophage Chemoattractant Protein-1 in Macrophage Recruitment , Angiogenesis , and Survival in Human Breas. *Clinical Cancer Research*, *6*(August), 3282–3289.

Utsunomiya, T., Hara, Y., Kataoka, A., Morita, M., Arakawa, H., Mori, M., & Nishimura, S. (2002). Cystatin-like metastasis-associated protein mRNA expression in human colorectal cancer is associated with both liver metastasis and patient survival. *Clinical Cancer Research*, *8*, 2591–2594.

Van Rhenen, A., Feller, N., Kelder, A., Westra, A. H., Rombouts, E., Zweegman, S., … Schuurhuis, G. J. (2005). High stem cell frequency in acute myeloid leukemia at diagnosis predicts high minimal residual disease and poor survival. *Clinical Cancer Research*, *11*(18), 6520–6527. https://doi.org/10.1158/1078-0432.CCR-05-0468

Van Rhenen, A., Moshaver, B., Kelder, A., Feller, N., Nieuwint, a W. M., Zweegman, S., … Schuurhuis, G. J. (2007). Aberrant marker expression patterns on the CD34+CD38- stem cell compartment in acute myeloid leukemia allows to distinguish the malignant from the normal stem cell compartment both at diagnosis and in remission. *Leukemia : Official Journal of the Leukemia Society of America, Leukemia Research Fund, U.K*, *21*(8), 1700–7. https://doi.org/10.1038/sj.leu.2404754

Varnum-Finney, B., Xu, L., Brashem-Stein, C., Nourigat, C., Flowers, D., Bakkour, S., … Bernstein, I. D. (2000). Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nature Medicine*, *6*(11), 1278–1281. https://doi.org/10.1038/81390

Vega, M. I., Jazirehi, A. R., Huerta-yepez, S., & Bonavida, B. (2005). Rituximab-Induced Inhibition of YY1 and Bcl-xL Expression in Ramos Non-Hodgkin's Lymphoma Cell Line via Inhibition of NF- B Activity: Role of YY1 and Bcl-xL in Fas Resistance and Chemoresistance, Respectively. *The Journal of Immunology*, *175*, 2174–2183. https://doi.org/10.4049/jimmunol.175.4.2174

Vogl, T., Gharibyan, A. L., & Morozova-Roche, L. A. (2012). Pro-inflammatory S100A8 and S100A9 proteins: Self-assembly into multifunctional native and amyloid complexes. *International Journal of Molecular Sciences*, *13*(3), 2893–2917. https://doi.org/10.3390/ijms13032893

Voigt, P., & Reinberg, D. (2013a). Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia The Cancer Genome Atlas Research Network. *The New England Journal of Medicine*, *368*(22), 2059–74. https://doi.org/10.1056/NEJMoa1301689

Voigt, P., & Reinberg, D. (2013b). Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia The Cancer Genome Atlas Research Network. *The New England Journal of Medicine*, *368*(22), 2059–74. https://doi.org/10.1056/NEJMoa1301689

Wander, S. A., Levis, M. J., & Fathi, A. T. (2014). The evolving role of FLT3 inhibitors in acute myeloid leukemia: quizartinib and beyond. *Therapeutic Advances in Hematology*, *5*(3), 65–77. https://doi.org/10.1177/2040620714532123

Wang, D., & Dubois, R. N. (2010). Eicosanoids and cancer. *Nature Reviews. Cancer*, *10*(3), 181–93. https://doi.org/10.1038/nrc2809

Wang, J. C. Y., & Dick, J. E. (2005). Cancer stem cells: Lessons from leukemia. *Trends in Cell Biology*, *15*(9), 494–501. https://doi.org/10.1016/j.tcb.2005.07.004

Wang, Y.-H., & Scadden, D. T. (2015). Harnessing the apoptotic programs in cancer stem-like cells. *EMBO Reports*, *16*(9), 1084–1098. https://doi.org/10.15252/embr.201439675

Wang, Y., Krivtsov, V., A., Sinha, A. U., North, T. E., Goessling, W., … Armstrong, S. A. (2010). The Wnt/b-Catenin Pathway Is Required for the Development of Leukemia Stem Cells in AML. *Science*, *327*(March), 1650–1653.

Ward, P. S., Patel, J., Wise, D. R., Abdel-Wahab, O., Bennett, B. D., Coller, H. A., … Thompson, C. B. (2010). The Common Feature of Leukemia-Associated IDH1 and IDH2 Mutations Is a Neomorphic Enzyme Activity Converting alpha-Ketoglutarate to 2-Hydroxyglutarate. *Cancer Cell*, *17*(3), 225–234. https://doi.org/10.1016/j.ccr.2010.01.020

Warner, J. K., Wang, J. C. Y., Hope, K. J., Jin, L., & Dick, J. E. (2004). Concepts of human leukemic development. *Oncogene*, *23*(43), 7164–77. https://doi.org/10.1038/sj.onc.1207933

Weiss, L., & Geduldig, U. (1991). Barrier cells: stromal regulation of hematopoiesis and blood cell release in normal and stressed murine bone marrow. *Blood*, *78*(4), 975–90.

Wenger, F. A., Kilian, M., Bisevac, M., Khodadayan, C., Von Seebach, M., Schimke, I., … Müller, J. M. (2002). Effects of celebrex and zyflo on liver metastasis and lipidperoxidation in pancreatic cancer in Syrian hamsters. *Clinical and Experimental Metastasis*, *19*(8), 681–687. https://doi.org/10.1023/A:1021387826867

Woelich, S. K., Braun, J. T., Schoen, M. W., Ramlal, R., Freter, C. E., Petruska, P. J., & Lionberger, J. M. (2017). Efficacy and Toxicity of Induction Therapy with Cladribine, Idarubicin, and Cytarabine (IAC) for Acute Myeloid Leukemia. *Anticancer Research*, *37*(2), 713–718. https://doi.org/10.21873/anticanres.11368

Woszczek, G., Chen, L.-Y., Nagineni, S., Kern, S., Barb, J., Munson, P. J., … Shelhamer, J. H. (2008). Leukotriene D(4) induces gene expression in human monocytes through cysteinyl leukotriene type I receptor. *The Journal of Allergy and Clinical Immunology*, *121*(1), 215–221.e1. https://doi.org/10.1016/j.jaci.2007.09.013

Wouters, B. J., & Delwel, R. (2016). Epigenetics and approaches to targeted epigenetic therapy in acute myeloid leukemia. *Blood*, *127*(1), 42–53. https://doi.org/10.1182/blood-2015-07-604512.Modes

Yang, L., Yang, M., Zhang, H., Wang, Z., Yu, Y., Xie, M., … Cao, L. (2012). S100A8-targeting siRNA enhances arsenic trioxide-induced myeloid leukemia cell death by down-regulating autophagy. *International Journal of Molecular Medicine*, *29*, 65–72. https://doi.org/10.3892/ijmm.2011.806

Ye, H., Adane, B., Khan, N., Sullivan, T., Minhajuddin, M., Gasparetto, M., … Jordan, C. T. (2016). Leukemic Stem Cells Evade Chemotherapy by Metabolic Adaptation to an Adipose Tissue Niche. *Cell Stem Cell*, *19*(1), 23–37. https://doi.org/10.1016/j.stem.2016.06.001

Ye, M., Iwasaki, H., Laiosa, C. V., Stadtfeld, M., Xie, H., Heck, S., … Graf, T. (2003). Hematopoietic stem cells expressing the myeloid lysozyme gene retain long-term, multilineage repopulation potential. *Immunity*, *19*(5), 689–699. https://doi.org/10.1016/S1074-7613(03)00299-1

Yu, C., Rahmani, M., Conrad, D., Subler, M., Dent, P., & Grant, S. (2003). The proteasome inhibitor bortezomib interacts synergistically with histone deacetylase inhibitors to induce apoptosis in Bcr / Abl ∂ cells sensitive and resistant to STI571, *102*(10), 3765–3774. https://doi.org/10.1182/blood-2003-03-0737.Supported

Zarini, S., Gijón, M. A., Ransome, A. E., Murphy, R. C., & Sala, A. (2009). Transcellular biosynthesis of cysteinyl leukotrienes in vivo during mouse peritoneal inflammation. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(20), 8296–301. https://doi.org/10.1073/pnas.0903851106

Zhang, Y., Gao, Y., Zhang, G., Huang, S., Dong, Z., Kong, C., … Huang, B. (2011). DNMT3a plays a role in switches between doxorubicin-induced senescence and apoptosis of colorectal cancer cells. *International Journal of Cancer*, *128*(3), 551–561. https://doi.org/10.1002/ijc.25365

Zhu, D., & Uckun, F. (2000). Cathepsin inhibition induces apoptotic death in human leukemia and lymphoma cells. *Leukemia & Lymphoma*, (612).

Ziboh, V. a., Wong, T., Wu, M. C., & Yunis, a. a. (1986). Modulation of colony stimulating factor-induced murine myeloid colony formation by S-peptido-lipoxygenase products. *Cancer Research*, *46*(February), 600–603.

Zlotnik, a, & Yoshie, O. (2000). Chemokines: a new classification system and their role in immunity. *Immunity*, *12*(2), 121–127. https://doi.org/10.1016/S1074-7613(00)80165-X

Zojer, N., Kirchbacher, K., Vesely, M., Hübl, W., & Ludwig, H. (2006). Rituximab treatment provides no clinical benefit in patients with pretreated advanced multiple myeloma. *Leukemia & Lymphoma*, *47*(6), 1103–9. https://doi.org/10.1080/10428190600564803

Zuber, J., Radtke, I., Pardee, T. S., Zhao, Z., Rappaport, a R., Luo, W., … Lowe, S. W. (2009). Mouse models of human AML accurately predict chemotherapy response. *Genes Dev*, *23*, 877–889. https://doi.org/10.1101/gad.1771409